# PHYSIOLOGICAL REVIEWS

VOLUME 26

1946

# CONTENTS


No 1 January, 1946

Histochemical Contributions to Physiology *Edward W Dempsey and George B Wislocki* 1

The Coronary Circulation *Donald E Gregg* 20

Recent Experimental Studies on Leukemia *J Furth* 48

Neural Mechanisms of Cutaneous Sense *Geo H Bishop* 78

The Absorption of Triglyceride Fat from the Intestine *A C Frazer* 104

The Use of Isotopically Marked Carbon in the Study of Intermediary Metabolism. *John M Buchanan and A Baird Hastings* 121

Yawning and Associated Phenomena *A Price Heusner* 157

No 2 April 1946

Sympathomimetic Amines: The Relation of Structure to Their Action and Inactivation *Karl H Beyer* 169

The Fixation of Carbon Dioxide and the Inter Relationships of the Tricarboxylic Acid Cycle *Harland G Wood* 198

The Effect of Body Temperature on Drug Action *Frederick A Fuhrman* 247

Lipins and Lipidoses. *S J Thannhauser and Gerhard Schmidt* 275

No 3 July 1946

Chemotaxis in Leukocytes *Morton McCutcheon* 310

The Physiology of Nervous Systems of Invertebrate Animals *C Ladd Prosser* 337

The Pharmacology of the Veratrum Alkaloids *Otto Krayer and George H Acheson* 383

Arthropod Nervous Systems A Review of Their Structure and Function *John H Welsh and William Schallek* 447

No 4 October 1946

Biotin and the Avidin Biotin Complex. *Roy Hertz* 479

The Metabolism and Permeability of Normal Skin *Herbert O Calvery John H Draize and Edwin P Laug* 495

Mechanism of the Development of Obesity in Animals with Hypothalamic Lesions *John R Brobeck* 541

Carbonic Anhydrase in Tissues other than Blood *Horace W Davenport* 560

Preparation and Chemistry of Anterior Pituitary Hormones *Abraham White* 574

Organization of the Respiratory Center *Robert F Pitts* 609

PHYSIOLOGICAL REVIEWS
VOL. 26 JANUARY, 1946 No 1

# HISTOCHEMICAL CONTRIBUTIONS TO PHYSIOLOGY

EDWARD W DEMPSEY AND GEORGE B WISLOCKI
*Department of Anatomy Harvard Medical School, Boston*

Histochemistry deals with the chemical characterization of substances in their natural locations within cells and tissues As Gersh (1941) has pointed out in an admirable review of the subject, histochemistry differs from conventional biochemistry in its emphasis upon the morphological localization of the various components which collectively comprise the body substance

Histophysiology relates the appearance of tissues, either in living states or in fixed and stained preparations, with different phases of functional activity The principal aim of histophysiology, as opposed to physiology in general, is to provide a morphological basis for the understanding of functional events

To follow this line of reasoning one step further, physiological histochemistry, the discipline resulting from the fusion of histochemistry and histophysiology, has as its chief aim the morphological localization within tissues of the chemical components which are involved in physiological processes and the correlation of any changes in this chemical machinery with the different functional states of the tissues or organism In other words, it concerns itself with the ultimate description of physiological, biochemical and histological phenomena each in terms of the others In its broader aspects, therefore, physiological histochemistry derives value not only from physical and chemical procedures, but also from the understanding of histological staining reactions in chemical terms, since variations in these staining reactions have been or may be related in turn to physiological events In a similar fashion, histochemical observations correlated with functional states may serve to illuminate morphological problems

In the present review we have brought together the diverse histophysiological, histochemical, and histological information available for a few situations We have made no attempt at exhaustive coverage, but have purposely limited ourselves to a small series of topics for which numerous data derived from different fields are available for correlation We hope, by the presentation of selected examples, to illustrate how the application of histochemical and cytochemical procedures can provide data of importance for understanding the physiological process underlying cellular activity We hope, also, to illustrate how the various aspects of such a correlated study reinforce and validate each other so that by the combined weight of the various lines of evidence conclusions may be drawn when a single approach would prove to be indecisive

GENERAL HISTOLOGICAL STAINING METHODS In recent years emphasis in histochemical investigations has shifted toward the development of highly specific reactions for individual compounds or classes of substances This trend is expressed in Lison's excellent monograph, *Histochemie animale* (1936), in which the statement is made repeatedly that certain reactions are valueless

to histochemistry because of their lack of specificity Although, other things being equal, it is certainly true that highly specific reactions are preferable to non-specific ones, the present reviewers would like to affirm their belief that relatively nonspecific histochemical reactions which nevertheless reveal a degree of morphological localization are often of considerable value from a chemical point of view It may be remarked parenthetically that chemists do not insist upon such extreme specificity For example, flow birefringence, a phenomenon which is certainly not specific for any chemical grouping since it depends rather upon the axial asymmetry of the molecule, has nevertheless been used effectively in physical chemistry A similar lack of specificity attaches to electrophoretic mobility, yet the present popularity of the Tiselius cell attests to the usefulness of the method for the solution of certain purely chemical problems It seems axiomatic that any method which faithfully produces differential results in different regions of tissues or cells does so because of chemical or physical differences in those regions These differences which are then responsible for the usual staining reactions of various dyes must ultimately be explained in histochemical terms Indeed, to ignore this precept is to neglect a most important tool It has been pointed out above that the principal emphasis in histochemistry should be upon the chemical characterization of morphological elements The classical histological methods, on the other hand, have been devised in the main to produce the sharpest possible differential results with reference to morphological and tinctorial appearances Obviously, therefore, the reconciliation of the specific, but frequently morphologically indistinct, reactions of histochemistry with the spatially precise, yet semi-specific, results of histology strengthens and sharpens the conclusions which can be drawn from both procedures Moreover, from a practical standpoint, the greatest promise exists for a rapid expansion of histochemistry because there is already a vast accumulated literature dealing with the careful correlation of functional states with histological appearances An interpretation of the conventional staining procedures in chemical terms would therefore permit a reinterpretation and extension of all of the histophysiological results presently available

Desirable as such an interpretation of staining methods in chemical terms may be, the path to that end is fraught with many difficulties From a chemical point of view the procedures which have been developed by histologists and cytologists, involving as they do fixation, dehydration, imbedding, clearing, mordanting, staining and differentiation, are so complex and frequently so contradictory as almost to defy chemical analysis Indeed, it is precisely this complexity that has caused many histochemists to seek chemically simpler, though morphologically less satisfactory, methods Nevertheless, difficult as histochemical insight may be, it is not necessarily impossible to attain Recent advances in protein chemistry on the one hand and histochemistry on the other, as we shall attempt to unfold, offer promise of progress in this difficult field

A great majority of histological procedures involve staining with a basic dye and counterstaining with an acid dye The significance of the observed attachment of these two dyes to the various components of tissues has long been

obscured by a controversy as to whether the dye-substrate combination involved chemical forces or physical bonds On the one hand, it has repeatedly been demonstrated that acidic and basic dyes react stoichiometrically with basic and acidic substances respectively On the other hand, nonionizable substances frequently stain intensely, particularly with basic dyes However, the solution to this particular controversy now appears to involve only the meaning of the particular terms Colloidal chemistry is apparently governed by forces similar to those involved in general chemistry but modified into a special case as they operate on surfaces The sign and magnitude of the surface charge differ in different physical-chemical conditions (Dubos, 1945) The charge determines the staining reaction, no matter whether the charge is due to the sum of ionic dissociations, to adsorbed ions, or to mixtures of these and other physical forces Thus, if we understand that it is charge rather than valence which we are measuring, the reaction of dyes with tissues assumes a physical-chemical meaning Acidophilia and basophilia, long recognized by histologists, thus appear to be designations capable of exact physical-chemical interpretation

The acidophilia or basophilia of tissues and cells is, however, not an invariant quality On the contrary, given structures may be selectively stained by either acid or basic dyes, depending upon the previous treatment of the tissues Two factors in particular profoundly modify the staining properties of histological materials These are the kind of fixation employed and the acidity of the medium in which the tissues are stained

Among the fixatives commonly employed in histology, formaldehyde occupies a prominent place Moreover, it has long been recognized that formaldehyde fixation renders proteins more acid and therefore increases their affinity for the basic dyes This change in basophilia is induced by the ability of formaldehyde to combine with free amino groups, thus preventing their dissociation and increasing the effective charge caused by the dissociated carboxyl groups of the protein On the other hand, heavy metal cations such as $Hg^{++}$, $Al^{+++}$, $Cu^{++}$, etc combine with the carboxyl groups of amino acids (Tolstoouhov, 1928, Dubos, 1945) and therefore increase the acidophilia of proteins

The effect of acidity and alkalinity upon staining reactions apparently can be accounted for satisfactorily by a consideration of ionic interchanges McCalla (1940, 1941a, 1941b) and McCalla and Clark (1941), working with bacterial cells, have demonstrated that adsorbed cations are displaced during staining by basic dyes If the experimental situation is arranged so that the adsorbed cations are hydrogen ions, the displacement of these ions by staining may readily be measured as a change in pH Acid dyes have a similar capability to displace anions from the absorption shell

The theory of ionic interchange also helps to explain some of the otherwise puzzling salt effects in staining McCalla (1941b) has shown that $Na^{+}$ and $K^{+}$ displace very little hydrogen, whereas ions such as $Pb^{+++}$, $Ag^{+}$, $Hg^{++}$ and $Al^{+++}$ displace a great deal The data submitted by McCalla also show that the activity of the heavy metal cation is not appreciably influenced by the kind of anion present in the system

These observations dealing with the physical chemical aspects of the staining reactions, although fragmentary and incomplete, are exciting in their implications From the standpoint of the conventional histological procedures, the results obtained by treatment with formaldehyde on the one hand and polyvalent heavy metals on the other are particularly significant The shift in the isoelectric zone of many proteins which is induced by these reagents apparently causes, at usual staining pH, basophilic or acidophilic staining depending on the reagent used Because of the affinity of formaldehyde for amino groups and of ions such as $Hg^{++}$ for carboxyl groups, we may assume that the proteins which show such lability in their staining reactions do so because their charges are predominantly determined by amino and carboxyl groups A corollary to this statement is that proteins the staining of which is relatively independent of the mode of fixation must depend upon other forces or more strongly dissociating groups for the determination of their charges We shall attempt to show, in the following sections, that such strongly dissociating groups do indeed characterize at least two groups of basophilic substances, the nucleoproteins and the mucoproteins

A *The nucleoproteins* The nucleoproteins are large molecules which contain a protein conjugated with nucleic acid The nucleic acid part of the nucleoprotein apparently determines its basophilic staining reaction, since after hydrolysis the resulting protein exhibits acidophilic properties Nucleic acids are in themselves complex substances composed of nucleotides which can in turn be broken down into component parts consisting of a purine or pyrimidine base, a sugar and phosphoric acid Two general classes of nucleic acids exist in cells, depending upon the kind of sugar incorporated into their structure One group, originally prepared from thymus tissue, contains a desoxyribose sugar and is frequently called desoxyribose nucleic or desoxyribonucleic acid The second type, originally prepared from yeast cells, contains a ribose sugar and is referred to as ribose nucleic acid or ribonucleic acid Nucleic acids are strongly charged compounds and have a great affinity for basic dyes Moreover, their basophilia is not destroyed by conjugation with proteins, so that the nucleoproteins are also strongly basophilic Excellent reviews dealing with the chemistry and occurrence of the nucleoproteins have recently been published by Mirsky (1943) and Greenstein (1944)

Although nucleoproteins have long been known to display pronounced basophilia and therefore to be among the substances which react with basic dyes in tissues, the positive identification of these compounds and their accurate localization in cells have only recently been accomplished Three methods have been developed which are of the utmost utility in this regard These are the staining method of Feulgen and Rossenbeck (1924) for desoxyribonucleoprotein, the use of ribonuclease, developed by Dubos (1937) and Brachet (1940) and subsequently improved after the enzyme had been prepared in crystalline form by Kunitz (1940), and the utilization of the specific ultraviolet absorption of nucleoproteins by Caspersson (1940)

The Feulgen method consists of the application of the Schiff test for alde-

hydes after a partial hydrolysis of the nucleoprotein in warm acid Such gentle acid hydrolysis frees the aldehyde group of the desoxyribose sugar which then is identified by the Schiff reagent The test, if carefully applied according to the directions of Feulgen, is apparently specific for desoxyribonucleoprotein since to date no other compound has been shown to give the reaction and the sites at which positive reactions have been obtained are in all cases compatible with the view that the reactive substance is nucleoprotein in character (cf Stowell, 1945) Indeed, the reactions obtained by this method are confined entirely to the nucleus in animal and plant cells and to discrete nucleus-like bodies in bacterial cells (Dubos, 1945) Moreover, not all of the basophilic substances of nuclei are stained The nucleolus, as well as certain regions of the chromosomes which stain intensely with basic dyes and which have collectively been called "heterochromatin," is prominently unstained by the Feulgen procedure

The discovery and preparation of an enzyme capable of depolymerizing ribonucleic acid into its constituent nucleotides paved the way for the cytological localization of ribonucleoproteins Dubos (1937) found that the basophilia of bacteria, excepting the discrete nucleus-like bodies mentioned above, was abolished after incubation in a solution containing ribonuclease, and, following Kunitz' (1940) isolation and crystallization of the enzyme, repeated the observations with the crystalline substance Brachet (1940) described the ability of a crude preparation of ribonuclease to abolish the characteristic cytoplasmic basophilia in a variety of animal cells, including those from the pancreas, intestinal epithelium, liver and nerve cells. Deschin (1940) reported that the basophils of the anterior pituitary gland lose their affinity for basic dyes after incubation in a solution containing ribonuclease Gersh and Bodian (1943) identified ribonucleoprotein in the Nissl bodies of nerve cells from the anterior horn of the spinal cord by the observation that these bodies lost their characteristic basophilia after digestion in a solution of crystalline ribonuclease Also using the crystalline enzyme, Dempsey and Wislocki (1945) destroyed the basophilic reaction of the syncytium and cytotrophoblast of the human placenta and of the basophilic cells in the anterior pituitary gland of the rat Davidson and Waymouth (1944) have recently identified basophilic inclusions in hepatic cells by means of digestion with crystalline ribonuclease

In addition to the cytoplasmic structures listed above, a number of investigators including Brachet (1940), Gersh and Bodian (1943) and Dempsey and Wislocki (1944) have noted the disappearance of basophilic staining in the nucleolus after treatment with ribonuclease

The development of ultraviolet microscopy and spectrophotometry by Caspersson and his collaborators has aided greatly our understanding of the localization of nucleoproteins These compounds exhibit strong absorption at about 2600 Å, due to the presence in the molecule of purine and pyrimidine components which also show strong absorption at 2600 Å However, although the intensity of absorption at this wave length may be used as a quantitative measure of the amount of these substances in fixed or living cells (Caspersson, 1940), the method does not discriminate between ribonucleoproteins and desoxyribonucleoproteins

since the absorption spectra of these two compounds are nearly identical Nevertheless, the fact that the ultraviolet method provides quantitative data is of the utmost importance, and, since qualitative discrimination between ribonucleoproteins and desoxyribonucleoproteins can be made by recourse to the Feulgen method and to treatment with ribonuclease, it is now possible to obtain reliable information on the changes in concentrations of both varieties of nucleoproteins at different functional stages Using these methods, Caspersson (1939) and Caspersson and Schultz (1940) have studied the ebb and flow of the nuclear nucleoproteins during the mitotic cycle, and Gersh and Bodian (1943) have observed the cycle of ribonucleoprotein disappearance and reappearance during retrograde chromatolysis and regeneration in the anterior horn cells of the spinal cord

The data presented above indicate that of all the basophilic inclusions in cells from the various tissues of the body, a considerable number owe their affinity for basic dyes to their content of nucleoproteins Moreover, it is now possible to distinguish between two types of nucleoprotein, the desoxyribonucleoproteins, which apparently are confined entirely to the nuclear chromatin, and the ribonucleoproteins which occur as characteristic cytoplasmic inclusions in many cells as well as in the nucleolus and other nuclear locations We shall attempt to show in the following sections that another large group of compounds, the mucoproteins, also exhibit basophilia and may be identified in cells by their cytological location and by their characteristic reactions

B *The mucoproteins* The mucoproteins are strongly acid, complex substances which on hydrolysis yield a protein, a hexosamine-carbohydrate and, as a rule, sulfuric acid Those which contain sulfuric acid are therefore to be regarded as sulfuric esters of glycoproteins and are strongly basophilic in their staining reactions They are either soluble or exhibit pronounced swelling in water, and they are easily hydrolyzed to free their constituent carbohydrate molecules These properties have been used with considerable success in devising methods for their identification

The strong acidity of mucus, apparently caused by the presence of sulfuric acid, accounts for the marked basophilic properties of the compounds Indeed, the basophilia exhibited by these compounds may be revealed even after staining in dye solutions so dilute that nuclear chromatin is unstained (Hempelmann, 1940) Although the mucoproteins have a great affinity for basic dyes, their basophilia exhibits a peculiar quality in that the tint assumed by the stained structure may be different from that of the free stain This property, usually referred to as metachromasia, is especially noteworthy with some of the thiazin dyes such as toluidin blue and polychrome methylene blue The phenomenon has been extensively studied from a histochemical viewpoint by Lison (1935) who regards metachromasia as indicative of a sulfuric ester of high molecular weight, since in no other class of compounds could the effect be observed Michaelis (1944) has adduced evidence that the phenomenon is caused by a mechanism whereby the dye is attached in a peculiar spatial pattern

Hempelmann (1940) has recently published the results of an excellent study

of the staining reactions of the mucoproteins To determine the specificity of the metachromatic reaction after staining with toluidin blue or polychrome methylene blue, he stained numerous sections of a wide variety of tissues with dilute solutions of these dyes The tissues in which the red violet metachromatic reaction was observed were *1*, the intracellular matrix of cartilage, *2*, the walls of arteries that had undergone mucoid degeneration, *3*, the ground substance of heart valves and some of the fibers of the atrioventricular ring, *4*, the sclera of the eye, *5*, tendon, *6*, intracellular mucus and the secretion of the epithelial mucous glands, *7*, Wharton's jelly in the umbilical cord, and *8*, the mucoid substance in egg yolk. To this list should be added the granules of mast cells and basophilic leucocytes (Holmgren and Wilander, 1940), certain decidual cells in the maternal placenta (Asplund, Borell and Holmgren, 1940) and the mesenchymal stroma of embryos (Holmgren, 1940) This list includes practically all of the tissues from which mucoproteins have been extracted chemically

In addition to the selectivity for mucoproteins which is displayed by the metachromatic reaction, Hempelmann found that two varieties could be differentiated on the basis of their affinities for dilute solutions of the two dyes When a solution of either dye diluted to 1 250,000 was used, the glandular mucus as well as the mucoprotein associated with the connective tissues was stained vividly When greater dilutions (up to 1 1,280,000) were employed, the epithelial mucus failed eventually to take the stain while the mesenchymal mucoproteins stained almost as intensely as before These two types of mucus differ in that epithelial mucus contains mucoitin sulfuric acid and a protein, whereas mesenchymal mucus is a complex containing chondroitin sulfuric acid In order to explain the differential staining at great dilution, it is necessary to assume that the chondroitin sulfuric protein has a greater affinity for the dye than does the mucoitin complex.

The ready solubility of mucus in water prevents its preservation in many histological fixatives Mixtures containing alcohol are consequently recommended For the best demonstration of the metachromasia of mucous substances, fixatives containing the salts of heavy metals are advantageous Jorpes, Holmgren and Wilander (1937), Holmgren and Wilander (1940) and Holmgren (1940), who have made extensive studies on the metachromatic granules of mast cells and on the occurrence and distribution of metachromasia in the tissues of adults and embryos, have relied for the most part on basic lead acetate as a fixative

In addition to staining metachromatically, the mucoproteins may be identified by tests which reveal the carbohydrate moiety of the molecule The carbohydrate is easily unmasked by mild hydrolysis and may then be stained in a number of ways Thus Schiff's reagent for aldehydes gives an intense purple reaction Since this reagent also stains glycogen (Bauer, 1932, 1933), the mucoprotein carbohydrate moiety must be differentiated from the former either by reference to the relative solubilities of the two compounds after fixation or, better still, by the saliva test which destroys glycogen but leaves the carbohy-

drate of mucoproteins unaffected The silver reduction test for glycogen employed by Mitchell and Wislocki (1944) also gives positive results for mucoproteins after suitable fixation Here again glycogen and mucoproteins may be differentiated by means of the saliva test Still another reaction which depends also upon the carbohydrate part of the mucus molecule is the test for glucosamine originally developed by Ehrlich (1901) This test involves the formation of a red color with dimethyl-paramido-benzaldehyde after mild alkaline hydrolysis This test is superior to the tests for reducing agents listed above in that it selectively reacts with glucosamine (Mann, 1902)

The mucoproteins, therefore, represent a class of compounds which exhibit strong affinities for the basic dyes They are frequently poorly preserved in histological sections unless specific attention is given to their fixation If adequately fixed, they may easily be recognized by their metachromasia after staining with certain dyes Gentle acid or alkaline hydrolysis splits them to form reducing bodies which give the characteristic reactions of glucosamine

C *Other basophilic inclusions* In addition to the cytoplasmic inclusions which have been sufficiently investigated to permit their identification as nucleoproteins or mucoproteins, a variety of other basophilic structures exists the nature of which is undetermined at present For example, the thyroid colloid shows strong basophilia, the intensity of which varies in different physiological states Although nucleoprotein is known to exist in the colloid (Salter, 1940), it remains to be determined whether this substance accounts completely for the basophilic reaction Finally, attention should be called to the fact that certain structures exhibit basophilia although auxiliary tests have shown neither nucleoprotein nor mucoprotein to be present The cross-striations of skeletal muscle are noteworthy in this regard These structures stain intensely with basic dyes after fixation in either formaldehyde or in salts of heavy metals, their staining reaction is not metachromatic, they do not exhibit a positive Feulgen reaction, and digestion with ribonuclease does not affect their affinity for basic dyes It must be concluded from these characteristics that the substance responsible for the basophilia of the striations does not depend for its staining characteristics upon the presence of carboxyl groups, and that it is not mucoprotein, desoxyribonucleoprotein or ribonucleoprotein

D *General considerations relating to basophilia* The considerations presented in the preceding sections point to the possibility that the generalized histological phenomenon of basophilia may be broken down into several categories depending upon specific histochemical reactions Moreover, the simultaneous comparison of the histological and histochemical preparations provides precision of localization with chemical specificity

It seems reasonable to divide the basophilic proteins into two main classes The first class consists of proteins, the isoelectric point of which is shifted to the alkaline side of the ordinary staining pH by fixation with the salts of heavy metals and to the acid side by fixation in formaldehyde The known combining powers of these fixatives make the assumption reasonable that these changes in isoelectric points are caused for the most part by rendering the carboxyl and amino groups, respectively, non-dissociable

The second class consists of proteins in which the isoelectric point is more acid than the staining pH after reaction with the salts of heavy metals Such behavior indicates the presence in the molecule of a strongly acid group, and in the case of two groups of proteins which fall within this class, strongly acid groups are known to be present Thus the nucleoproteins are phosphoric esters and the mucoproteins are sulfuric esters It should, however, be pointed out that the present information does not preclude the presence of other basophilic groups in these molecules For example, it is quite likely that fixation by heavy metals shifts the isoelectric point of nucleoproteins in the alkaline direction, albeit not so far as to reach the pH at which histological staining reactions are ordinarily carried out

These considerations point a direction for two future lines of research. If valid, they indicate that strongly acid radicals should characterize the still poorly understood basophilic compounds such as those responsible for the basic staining of the cross-striations of skeletal muscle In this connection, it is well known that a variety of phosphate-containing substances such as phosphocreatine and adenosine triphosphate is present in muscle and are importantly concerned with the chemistry of muscle contraction The second, more general, line of research involves the characterization of histologically demonstrable proteins by determining their isoelectric points and by the displacement of these isoelectric points by treatment with reagents such as formaldehyde, heavy metal ions, etc Hence, determining the pH at which a given cellular inclusion changes from basophilic to acidophilic staining should provide data for comparison with similar information derived from the study of purified compounds in vitro

Reactions of lipoidal materials Both the histochemical localization and identification of fat-soluble substances are of considerable importance for the study of cellular physiological processes The absorption, transport, storage and metabolism of fats all pose questions involving accurate localization and identification Moreover, a number of lipoidal compounds of biological importance are now known (e g , the steroid hormones and the fat-soluble vitamins) Finally, modern views of cellular organization envision the presence of protoplasmic interfaces on which surface reactions may occur and lipoids are apparently of great importance in the production of such interfacial surfaces

Among the general methods for staining lipoids, two procedures have been quite popular These are the reduction methods in which silver or osmic acid is reduced to a black precipitate, and the solubility methods in which the fatty materials are colored by a fat-soluble but water insoluble dye The reduction methods are capricious and non-specific, but, on the other hand are exquisite in the morphological detail which they reveal The solubility methods which usually employ Sudan III or Sudan IV produce somewhat less precise localization but provide information on the solubility characteristics of the structures which are stained Moreover, in recent years the use of a new dye, Sudan black B, has overcome some of the limitations of the earlier procedures It has long been known that the lower members of the Sudan series do not stain all lipoids, since they are relatively insoluble in such lipoidal substances as the phospholipids and cholesterol In addition, the color of the red Sudan dyes is not suffi

ciently intense to permit resolution of the smallest lipoidal inclusions Sudan black, however, generates an intense stain in myelin, in the Golgi apparatus, and in other lipoidal inclusions which are not revealed by the lower members of the Sudan series (Baker, 1944)

Accurate chemical characterization of the lipoidal materials in microscopic sections is not yet possible Nevertheless, a beginning at least has been made and several group reactions are now known The Smith-Dietrich test for lipines (Dietrich, 1910) is apparently one of these (Lison, 1936) Although the test is not absolutely specific in a chemical sense, compounds such as lecithin, cephalin and sphingomyelin resist the differentiation imposed in the test to such a degree that a positive reaction is very strong presumptive evidence for their presence Baker (1944) has recently adduced evidence from this and other reactions that a nitrogenous lipoid is present in the Golgi apparatus of several diverse cells

One of the reactions which characterizes true fats, as well as other lipoidal substances, is saponification This reaction, which results in the formation of soaps, does not appear to have received the attention it deserves In plant tissues, however, the formation of soaps by alkaline hydrolysis of fats has proved useful (McClung, 1937) Perhaps the reason for its present disrepute stems from the erroneous belief of earlier investigators that the method differentiated free fatty acids from their esters (Fischler, 1904) This it certainly does not do (cf Lison, 1936, pp 203–204) Nevertheless, a classification of fatty inclusions based upon the ease or impossibility of their saponification should prove useful, particularly in tissues in which both saponifiable and non-saponifiable lipoids are simultaneously present The method should also prove useful for removing saponifiable material from sections leaving the non-saponifiable fraction for subsequent study

The recent development of the related fields of steroid chemistry and endocrinology has prompted several investigations of the various glands which secrete the steroid hormones These hormones are all alike in that they contain a cyclopentenophenanthrene nucleus The various compounds which have diverse biological effects differ in the kind and location of various side groupings on the nucleus The activity appears to be due to hydroxy- or keto-groupings, for the most part Reasoning that ketone reactions, coupled with solubility tests, should reveal the location of the ketosteroids, Bennett (1940) found that the outer layer of the fasciculata in the adrenal cortex of the cat contained acetone-soluble materials which formed phenylhydrazones and semicarbazides He showed also that the lipoids in this area were capable of reducing silver in alkaline solutions The lipoidal droplets contained within the cells were frequently birefringent, and, on reaction with digitonine, anisotropic crystals were formed Since these reactions are characteristic of ketosteroids, and since they were negative in regions of the adrenal cortex other than the outer layer of the fasciculata, Bennett concluded that this zone represents the site of origin and secretion of the adrenal cortical hormones Subsequently, the phenylhydrazine reaction has been applied to the mammalian testis where

phenylhydrazones are formed in the interstitial cells (Pollock, 1942), to the rat's ovary, where the reacting substances are restricted to thecal, luteal and interstitial cells (Dempsey and Bassett, 1943) and to the human placenta in which reactions have been observed in the syncytial trophoblast (Wislocki and Bennett, 1943)

Bennett's conclusions were severely criticized by Gomori (1942) who pointed out that the phenylhydrazones formed after treatment with phenylhydrazine were superimposable upon the results obtained by the plasmal reaction of Feulgen and Voit (1924) The plasmal reaction involves the recolorization of leucofuchsin, which, under the name of Schiff's reagent, is commonly employed in tests for aldehydes Feulgen and Voit (1924), Verne (1929), and Gomori (1942) regard the reaction as indicating the presence of aldehydes of fatty acids. However, it has not been possible to oxidize fatty acids by oxidations as mild as those which are effective in the plasmal reaction, and Lison (1932) in a comprehensive study found that positive reactions with Schiff's reagent could be obtained with aldehydes, certain ketones, and even by some unsaturated compounds which contained no carbonyl grouping Moreover, the carbonyl group in certain steroids is active enough to react with phenylhydrazine (Bennett, 1940), Schiff's reagent (Dempsey and Wislocki, 1944), and silver in alkaline solution (Reichstein and Shoppee, 1943) It therefore appears that these reactions, commonly employed to detect aldehydes, are not capable of differentiating between aldehydes and ketosteroids Yet, accessory evidence to be presented below, indicates that at least in the adrenal gland, ovary, testis and placenta, positive phenylhydrazine reactions occur in the same locations which also may exhibit other steroid reactions and are therefore indicative of steroid substances

Among the characteristic reactions of the steroid hormones, their fluorescence (Bierry and Gouzon, 1936, Reichstein and Shoppee, 1943), their ability to form spherocrystals, their reaction with digitonine to form crystals which are anisotropic (Fieser, 1937), and their response to the Liebermann Burchardt test are noteworthy These procedures have been applied in this laboratory to the ovary (Dempsey and Bassett, 1943) and the placenta (Dempsey and Wislocki, 1944) In brief, positive reactions have been obtained in the same locations which also contain substances reacting with phenylhydrazine and Schiff's reagent

Since the active hormones elaborated by the organs studied are steroids possessing keto-groups, it seems reasonable to attribute both the positive steroid reactions and the reactions with phenylhydrazine and Schiffs reagent to the hormones or their precursors At all events, the point of doing these tests is to locate the areas of the glands which contain biologically active substances Even if some of the positive areas contain additional substances such as aldehydes which add to the intensity of reaction, or if the adventitious aldehydes turn out to be more widely distributed than the hormones themselves, it seems that one can safely say that the non reactive areas do not contain the hormones in significant amounts

The correlation of the steroid reactions described above with different physi-

ological states has so far been done only fragmentarily Sarason (1943) has reported that the sudanophilic and birefringent zone in the adrenal cortex decreases in thickness after hypophysectomy The changes in the adrenal cortex during the alarm reaction have been studied by Popjak (1944) In moderate stress there is an increase in the phenylhydrazine reaction, whereas during severe stress a depletion of the substances which form phenylhydrazones occurs These results have been confirmed in this laboratory (Deane, unpublished experiments) for both hypophysectomized rats and rats subjected to cold environments The changes observed after these experimental procedures may be detected either with the Schiff reaction or by observing the birefringence or fluorescence of

[illegible] For the ovary of the rat, Dempsey and Bassett (1943) described the [illegible] the various reactions at approximately the time at which the Graafian [illegible] develops its antrum They also called attention to changes in the reac[illegible]which occur during the lifetime of the corpus luteum, but made no de[illegible] analysis of these changes in reference to the physiological cycle of the [illegible]y However, Everett (1945) has recently correlated the Liebermann[illegible]hardt reaction with the stage of the reproductive cycle in rats and has [illegible]wn that a marked change in the reactiveness of the corpus luteum of the previous cycle normally occurs just prior to ovulation In persistently estrous rats, in which ovulation does not spontaneously occur, there is an almost complete deficiency in the reactive material of the corpus luteum Since Everett [illegible] previously shown that the persistently estrous rats fail to ovulate because of a preovulatory deficiency of progesterone, there is presumptive evidence that the Liebermann-Burchardt reaction may serve as an index of progesterone for[illegible]on and secretion This very promising lead should be investigated further

[illegible] far as the testis is concerned, relatively little is known Talbot and Demp[illegible] (unpublished observations) have found little or no birefringent lipoids in the [illegible]tial cells of infants and young boys, whereas considerable amounts are [illegible] in the Leydig cells of adult men In one case of marked sexual pre[illegible]ity, birefringent lipoids were observed in the testis of a child Conversely, birefringence of the testicular lipoids was decreased or absent in several cases of hypogonadism

Before passing from the subject of the steroid reactions, it may be appropriate to mention a valuable improvement on the phenylhydrazine hydrochloride method of Bennett which has been worked out in this laboratory by M Pechet, but which has not yet been published This method involves the use of 2-4 dinitrophenylhydrazine sulfate in phosphate buffer at pH 6 0–6 8 The phenylhydrazones formed with this compound are much deeper in color than are those formed after treatment with phenylhydrazine hydrochloride In consequence, thinner sections can be prepared and much better localization of the reactive substances is possible than following the previous method Moreover, the nitro-derivative reacts more energetically than does phenylhydrazine, a circumstance which has been utilized to advantage in identifying the less reactive carbonyl groupings in sections of the adrenal cortex by the combined use of the hydrochloride and the nitro-derivative The procedure devised involves treatment

of the section with phenylhydrazine hydrochloride until the more reactive groups are combined, followed by subsequent immersion in a solution of 2-4 dinitrophenylhydrazine sulfate The red dinitrophenylhydrazone may be differentiated from the yellow phenylhydrazone by virtue of the difference in their colors

Vitamin A has recently received considerable attention because of the possibility of localizing it by virtue of its specific fluorescence This subject has recently been reviewed by Popper (1944) However, the characteristic greenish fluorescence of vitamin A may be confused with the similar greenish fluorescence exhibited by many steroids Nevertheless, the two may be differentiated in a variety of ways The fluorescence of vitamin A fades rapidly on exposure to ultraviolet light, whereas the greenish fluorescence identifiable in the organs which secrete steroidal compounds is stable and remains unaltered after hours of exposure Moreover, the Carr-Price reaction, as modified for tissue sections (cf Cowdry, 1943), provides a color test which is specific for vitamin A Another chemical reaction which has so far not been applied to sections, has recently been published by Sobel and Werbin (1945) This test involves the development of a blue color when vitamin A reacts with glycerol 1,3-dichlorohydrin The technique is somewhat simpler than is that in the Carr Price reaction, the color is more stable, and carotene may also be detected as this latter substance produces a green color

Enzymatic reactions as applied to tissue sections The study of enzymes and enzymatic reactions has received increasing attention from biochemists in recent years It is now generally believed that most of the chemical reactions which occur in living cells are controlled by enzymes The enzyme exerts a directing influence by making the reaction proceed so rapidly in one direction that side reactions become of negligible importance (Sumner and Somers, 1943) It is apparent, therefore, that studies of the concentration and localization of enzymes provide information which can be related directly to the physiological activity of the tissues Unfortunately only very few of the numerous enzyme reactions in the armamentarium of the chemist are at present applicable as true histochemical reactions A certain number of microchemical procedures have been devised and have been carried out with ingenious histological control by the methods of Linderstrøm Lang (cf Biol Symp , 1944, Ann Rev Biochem , 1944 for references), but the degree of cytological localization possible with these procedures is actually very slight Among the methods which have successfully applied to sections, those concerning a variety of oxidases and peroxidases have received the greatest amount of attention

A. *Oxidative enzymes* The fact that organs differ in their oxidizing powers has been a part of biochemical knowledge since Ehrlich (1885), who showed that the injection of substances now described as reduction-oxidation indicators stained some organs but not others Among the reactions which he employed was the formation of indophenol blue upon injection of alpha naphthol and para phenylenediamine This reaction is usually described in current literature as the indophenol oxidase reaction, the nadi reaction, or simply as the oxidase reaction It has been applied to a large number of animal tissues In recent years

this reaction has attained even greater popularity since Keilin (1933) demonstrated that it reveals the presence of Warburg's *Atmungsferment*, now ordinarily described as the cytochrome oxidase-cytochrome c system

The indophenol oxidase reaction, although it apparently reveals the cytochrome system, must be carefully employed to give reliable results Substances occur, notably in cells of the myeloid series, which are responsive to the reaction after drying or other types of fixation On the other hand, more labile substances exist in the cells of many other tissues, the reaction in which can only be obtained in fresh unfixed tissues Moreover, the pH at which the reaction is carried out markedly influences the results Lison (1936) regards only the reaction carried out in alkaline solution on fresh tissues as indicative of the presence of the cytochrome oxidase-cytochrome c system

In addition to the nadi reaction, similar results have been obtained by the use of other phenolic substances as substrates for the enzyme system (Lison, 1936)

The oxidase reactions, as employed above, are capable of utilizing oxygen in its atmospheric or molecular form In contrast to the oxidase enzymes, another set of enzymes commonly known as the peroxidases, are capable of oxidizing the same substrates but only in the presence of nascent oxygen, the source of which is ordinarily hydrogen peroxide The presence of true peroxidases was formerly doubted in mammalian tissues, since the ubiquitous occurrence of catalase together with the extreme toxicity of peroxide was thought to preclude any biological importance of the peroxidase system However, it now seems well established that peroxidases are a normal constituent of mammalian cells (cf Lippman, 1943) and a tentative function has been suggested for the enzyme in the metabolism of iodine in the thyroid gland (Dempsey, 1944) In general, it may be said that the oxidase and peroxidase reactions reveal the specific activity of the respective enzymes This statement appears to be true by definition, since the test actually involves the catalytic coupling of oxygen with the various substrates Moreover, the results obtained by these reactions are in good agreement with those secured by the use of reduction-oxidation indicator dyes (cf Flexner and Stiehler, 1938) So far as histological localization is concerned, the reactions appear to be entirely adequate, since the colored oxidation products of the substrates are highly insoluble substances and are therefore precipitated out in the cells in which the enzyme occurs However, there is some question as to the cytological precision of the tests Lison (1936, p 278 et seq ) has reviewed this problem, a short résumé of the elements of which is discussed here In brief, the problem involves the possibility that elements exist within the cell which show an extreme affinity for the oxidation products of the substrate Hollande (1924) has presented experiments showing that identical effects can be obtained either by the nadi reaction or by staining with an alcoholic solution of indophenol blue, diluted at the moment of staining with water Such dilution of the dye causes it to assume a metastable condition from which it is easily precipitated The precipitation apparently occurs selectively upon certain intracellular granules, and this gives rise to the typically

granulated appearance characteristic of the nadi reaction. These granules, according to Hollande, are, properly speaking, not oxidase granules but indophenophile granules In the case of the peroxidase reaction in which benzidine is used as a substrate, the granules are oxybenzophile

Regardless of the degree of cytological precision possible with these methods, they provide, nevertheless, information which may be correlated with the functional state of individual tissues Flexner and Stiehler (1938) have observed that a marked increase in the oxidase reaction corresponds in time to the beginning of secretion by the choroid plexus of the pig embryo A similar situation obtains in the developing renal tubules (Flexner, 1939) Dempsey and Wislocki (1944), in studying the distribution of glycogen in the human placenta, noted that glycogen accumulation occurred in tissues the oxidase activity of which was low They also pointed out, from comparing Weed's (1917) data on glycogen storage in the cells of the developing choroid plexus with Flexner and Stiehler's (1938) information concerning the occurrence there of oxidase reaction and reaction oxidation potentials, that glycogen precedes the advent of the oxidase reaction, the two processes being apparently mutually incompatible A somewhat similar relationship exists in the developing renal tubules These considerations and others led Dempsey and Wislocki (1944) to postulate that the significance of glycogen storage in cells of the placenta and fetal organs might be to furnish energy for anerobic respiration in cells the respiratory metabolism of which was insufficient for their needs

Both the oxidase and peroxidase reactions have also been correlated with the activity of the thyroid gland Blue granules can easily be demonstrated in the thyroid cells after application of the nadi reagents The peroxidase, or benzidine-$H_2O_2$ reaction, also causes blue granules to appear in the thyroid cells The latter reaction is abolished by the addition of thiouracil to the bath in which the sections are stained, whereas the oxidase reaction is unaffected by this drug Since thiouracil prevents the formation of thyroid hormone by the thyroid gland (Astwood, Bissel, Sullivan and Tyslowitz, 1943, MacKenzie and MacKenzie, 1943) and since peroxidase catalyzes reactions analogous to those involved in thyroxin synthesis (Westerfeld and Lowe, 1942), Dempsey (1944) suggested that peroxidase was a natural component of the thyroid cell and that it participated in the reactions leading to the formation of thyroxin On the other hand, the reduction-oxidation potentials of the thyroid gland undergo changes in different physiological states (DeRobertis and Goncalves, 1945), and poisons, such as cyanide and azide which inhibit the cytochrome system, also inhibit the synthesis of thyroxin in surviving slices of thyroid tissue (Schachner, Franklin and Chaikoff, 1943) The latter authors have suggested, therefore, that the cytochrome system provides the oxidative energy for the synthesis of the thyroid hormone

B *The dopa oxidase reaction* An enzyme exists in melanin forming cells which is capable of catalyzing the oxidation of desoxyphenylalanine This substance, usually abbreviated to dopa polymerizes when oxidized to form a tarry compound similar to, if not identical with, the natural pigment (Bloch,

1917) The dopa reaction as carried out on tissue sections involves the incubation of the section in a solution of dopa The presence of dopa oxidase is indicated by the formation of a black or brown insoluble pigment at the site of the enzyme

In recent years interest in pigment formation has been revived because of the discovery of physiological methods for the control of melanogenesis Ralli and Graef (1943) have described changes in pigmentation induced by altering the adrenal cortical function of rats Similarly, graying of the hair has been described as a result of deficiency in certain B vitamins (cf Unna, Richards and Sampson, 1941) So far as the present reviewers are aware, however, systematic studies of the relation of the dopa reaction to the factors causing changes in pigmentation have not yet been made Such studies should contribute greatly to our understanding both of the mechanism of melanogenesis and the physiological effects of the vitamins and hormones on pigmentation

C *Phosphatases* The simultaneous publication in 1939 by Gomori and Takamatsu of nearly identical methods for the histological demonstration of alkaline phosphatase opened a new chapter in enzymatic histochemistry The methods involve the incubation of tissue sections in a buffered solution containing glycerophosphate as a substrate and calcium ions Under these conditions the enzyme liberates phosphoric acid from the phosphate The phosphoric acid immediately reacts with the calcium ions and, in an alkaline medium, precipitates out as tricalcium phosphate The latter substance is finally revealed by transformation into a black precipitate by treatment with cobaltous salts and ammonium sulfate (Gomori, 1941a), by staining with alizarin (Kabat and Furth, 1941), or by silver impregnation methods (Bourne, 1943)

Soon, following the publication of his method for alkaline phosphatase, Gomori (1941b) announced a similar method for the demonstration of acid phosphatase Since most inorganic phosphates are soluble in acid solution, it was necessary to find one which was insoluble at pH 4 7, the optimum of the enzyme Gomori found that lead phosphate was sufficiently insoluble to meet these requirements and that lead salts did not inhibit the reaction

The discovery of the two methods for alkaline and acid phosphatase led very shortly thereafter to the publication of three valuable survey papers (Gomori, 1941a, Kabat and Furth, 1941, Wolf, Kabat and Newman, 1943) in which the principal sites of phosphatase activity in the mammalian body were described It was immediately apparent from these studies that phosphatases were widespread in their occurrence in the body In various locations phosphatase occurred variously within cell nuclei, in the cell cytoplasm, in tissue spaces and in the lumina of glands However, when different species were compared one with another, marked differences in distribution and concentration of the enzymes became apparent For example, in the liver, the reaction occurs variably and unpredictably in the endothelial cells, the hepatic cells and the bile capillaries of different species Similarly, in the ovary of the rabbit, the granulosa cells contain alkaline phosphatase and the thecal cells none, whereas in the sow and monkey, the enzyme is restricted to the theca and does not occur in the granulosa (Córner, 1944)

Despite such apparent contradictions in different species, there is reason to believe that phosphatases are importantly concerned in various metabolic processes Thus, in developing bone, there is an alkaline phosphatase, the purpose of which is apparently to provide phosphate ions for the calcificatory process (Robison, 1922, Harris, 1932, Horowitz, 1942) Similarly, phosphatases are believed to play rôles in the metabolism of nucleotides, phospholipids and carbohydrates (Sumner and Somers, 1943) We do not wish to discuss these relationships fully here, but will return to the possible significance of phosphatase to these metabolic processes in a later section

Emmel (1945) has recently made the interesting observation that alkaline phosphatase is concentrated in the Golgi zone of the absorptive cells of the intestinal mucosa The amount of the enzyme present in the Golgi apparatus of these epithelial cells is so great that the phosphatase preparations rival those prepared by osmic acid methods in the elegance with which the Golgi apparatus is displayed We have confirmed Emmel's observation regarding alkaline phosphatase (Deane and Dempsey, unpublished data) and have made the additional observation that acid phosphatase is also prominent in the Golgi region of intestinal cells Moreover, both acid and alkaline phosphatase preparations differ in one interesting respect from the Golgi preparations made by osmic acid or silver reduction With these latter methods, the Golgi net is most prominently and easily impregnated in the cells covering the tips of the villi, whereas the cells lining the sides and deeper parts of the villi are more difficult to demonstrate (Weiner, 1928) Conversely, in the phosphatase preparations, the Golgi apparatus in the cells at the tips of the villi is never impregnated, whereas it is easily demonstrable in the cells at the sides and base Emmel was unable to demonstrate alkaline phosphatase in the Golgi region of kidney epithelium and therefore regarded the phosphatase reaction in the Golgi apparatus of the intestinal epithelium as unique. Nevertheless, although more difficult to achieve, we have been able to demonstrate both acid and alkaline phosphatase in the form of granules or even occasionally as well-defined nets in the zone occupied by the Golgi apparatus in a variety of epithelia including the liver, the kidney, the pregnant uterus, the seminal epithelium and the epididymis These observations afford a specific chemical clue to the significance of the Golgi apparatus On purely cytological grounds the Golgi apparatus has long been regarded as a center of vital cellular and secretory activity, partly because of its invariable enlargement in states of heightened physiological activity and partly because secretory granules appear to be formed in association with it or at least in its vicinity (Kirkman and Severinghaus, 1938) The observations cited above seem to substantiate this view on cytochemical grounds, indicating as they do a close association between the Golgi apparatus and enzymes known to participate importantly in cellular metabolic processes

An interesting example of how chemical inferences may be drawn from histological data is derived from the action of different fixatives upon phosphatase Gomori (1941b) remarks that phosphatase cannot be demonstrated in sections fixed in standard histological fixatives such as formaldehyde or mixtures which contain such substances as mercuric chloride or picric acid One can conclude

from these facts that for its proper action the enzyme requires both free amino and carboxyl groups, since, as we have already discussed in an earlier section, formaldehyde and the heavy metals used as fixatives have the properties of combining respectively with amino and carboxyl groups and preventing their dissociation Consequently, from a histochemical point of view, considerable interest attaches to a recent paper by Gould (1944) in which after purely chemical procedures it was concluded that phosphatase requires the presence of free amino and carboxyl groups

CHEMICAL INTERRELATIONSHIPS IN CELLS COMPARATIVE CYTOCHEMISTRY It is a well-recognized principle in histology that organs or tissues may be regarded as the sum of a relatively small number of different structural elements comprising the component cells and interstitial materials The morphological characteristics of the cells and interstitial materials serve to differentiate different tissues one from another For example, the principal distinction between bone and fibrous connective tissue might be expressed by the statement that the interstitial matrix of the former contains dense deposits of calcium salts whereas the latter does not A system of classification based upon these principles was, as a matter of fact, proposed by Oliver Wendell Holmes in 1862 together with the suggestion that suitable symbols might be attached to the various elemental morphological constituents so that histological structures could be represented by formulae similar to those which have proved useful in chemistry This suggestion never caught on, probably because the number of elemental components which could be agreed upon was so small as to make such symbolic representation unnecessary

At the cytological level of study, some systematization based principally upon similarities of morphological appearance and reactivity to staining procedures has been achieved in the past The classification of the cells of the organism into the component tissues of the body is such a systematization (Bichat, 1801) However, the available criteria are in most instances so coarse that only the crudest kinds of similarities are enjoyed by some of the members of the same group For example, the epithelia are so widely divergent from one another both in structure and apparent function that relatively little is to be gained by grouping the parenchymal cells of the thyroid with, say, those of the epidermis into one category Such discrepant aspects within the customarily recognized divisions have led to a kind of systematic empiricism shared by histologists and cytologists whereby only lip-service is paid to the category, and each organ or indeed each of several separate cell types which may occur within it is regarded as a separate and unique phenomenon

The introduction of histochemical and cytochemical methods permits another system of classification or characterization of cells It is possible, for example, to group into one category the cells in which basophilic cytoplasmic nucleoproteins are an outstanding characteristic, into another category those cells which contain large amounts of phosphatase, and into a third group those which exhibit glycogen storage This system is capable of indefinite expansion as other

methods reveal further histochemical properties Moreover, eventually it becomes possible to correlate such individual chemical characteristics with underlying important chemical processes within a given type of cell and one thereby begins to become acquainted with the cell in dynamic or functional terms

Examination of the proposal to divide cells into categories based on histochemical characterization reveals at once the importance of selecting significant chemical entities If, for example, one were to choose the presence merely of protein substances as a common denominator, the group would be too inclusive to be significant Ideally the distinguishing characteristic should be made sufficiently restrictive so that its presence would reveal a specific cellular activity As an example one could cite the presence of hemoglobin in erythrocytes Between the extremes cited there would exist a variety of characteristics of varying degrees of specificity and usefulness

The usefulness of such a procedure will depend, in the final analysis, upon the number and importance of the new facts which are brought to light and upon the future experiments which this manner of approach suggests We believe that such an approach relating to the chemical characteristics of diverse groups of cells should illumine many aspects of cellular biology Although at the present time our technical methods permit only the most fragmentary penetration into this field, we believe that further extension of histochemical methods and correlation will be exceedingly fruitful. We should like, therefore, to present in the remainder of this review a few examples of correlations between histochemical characteristics and cellular activities

A *The relation of nucleoproteins to protein synthesis* Greenstein (1944) has recently reviewed the evidence for nucleoprotein participation in protein synthesis and has discussed the possible mechanisms by which the nucleoprotein exerts its controlling influence It is consequently unnecessary here to dwell extensively upon the evidence supporting the hypothesis that nucleoproteins are enzymes which regulate protein formation or are templates upon which particular kinds of proteins are fashioned Instead, we prefer to summarize only briefly some of the information presented by Greenstein and to add a few accessory observations which have become available since his article was published

As discussed in an earlier section of this review, the cytoplasmic basophilia which is a characteristic feature of many different cells widely located throughout the body is now known to be caused by the presence of ribonucleoproteins Cells exhibiting cytoplasmic basophilia occur in most parts of the body and may be derived from any of the three germ layers, and since they contain nucleoproteins, it is logical to assume that they are engaged in rapid protein synthesis Furthermore, in certain instances, differences in the rate of protein formation are accompanied by differences in the amount or distribution of the cytoplasmic basophilia. We should particularly like to emphasize this relationship of nucleoprotein concentration to protein synthesis

The sero-zymogenic glands associated with the digestive system exhibit

basophilic staining in the basal portion of the cell Basal chromidial substance is a distinguishing feature of the serous portions of the salivary glands, the pancreatic acini and of certain of the glandular cells within the intestinal tract In these cells, the zymogen granules ordinarily occupy the apical portion of the cell extending downward so that in the resting gland the most basally located granules overlap the region occupied by the basal chromidial substance Upon stimulation of the gland to secretory activity the cells are readily depleted of their apically located granules whereas only rarely and after intense stimulation do the more basally located granules disappear (Babkin, 1944) Moreover, during secretory activity, the chromidial substance becomes more abundant and may be stained more intensely (cf Mirsky, 1943)

It is a well-known fact that the early developmental stages of the blood cells are typically basophilic In the case of the erythrocyte series, basophilia is inversely proportional to the content of hemoglobin within the cell Young stages are strongly basophilic and contain no hemoglobin, juvenile stages are characterized by diminishing basophilia and an increasing concentration of hemoglobin, whereas mature erythrocytes stain almost not at all with basic dyes and contain an abundance of hemoglobin A similar relationship obtains between basophilia and the specific granules of the myelocytes, as the granules increase the basophilia declines to complete extinction in the mature cells Consequently, in both the erythrocyte and the granulocyte series, basophilia declines inversely with the accumulation of the specific cytoplasmic proteins

The situation in regard to the lymphocyte is somewhat more complex and until recently was less well understood The cytoplasm of cells of the lymphocyte series is typically basophilic both in early and mature developmental stages However, the relationship of this fact to the possibility of protein synthesis has only recently been elucidated A brilliant series of investigations to which contributions have been made by Dougherty and White (1945), Harris, Grimm, Mertens and Ehrich (1945), Kass (1945), and Ehrich and Harris (1945) has demonstrated that during certain physiological states there is a mobilization of lymphocytes from their stores in the tissues, while at the same time in the blood stream a lymphopenia occurs This paradoxical situation is explainable on the hypothesis that the lymphocytes undergo liquefaction in the circulation, a corollary to this being that they liberate the proteins composing them into the blood stream Investigations designed to test this hypothesis have shown that parallel to the disappearance of the circulating lymphocytes there is an increase in the gamma globulin fraction of the plasma proteins, and that washed lymphocytes produce, when lysed, a protein the electrophoretic mobility of which is identical with that of the gamma globulin Furthermore, Ehrich and Harris (1945) have demonstrated that the lymphocytes contribute to antibody production when activated by antigens In consequence of these extraordinary findings, it now appears that a major function of lymphocytes is to produce globulins which participate in immune reactions Consequently, the globulin synthesis of the lymphocyte can now be related to the cytoplasmic nucleoproteins which are responsible for basophilic properties of the cells

The phenomenon of chromatolysis in nerve cells after section of the axon has long been known This process involves the dissolution and disappearance of the Nissl bodies during retrograde degeneration of the nerve fiber Subsequent to the degenerative chromatolysis, regeneration of the axon occurs and there is a parallel reconstitution of the Nissl substance For several reasons, including their basophilic property, Held as long ago as 1895 suggested that the Nissl bodies were nucleoprotein in nature However, not until recently was the fact firmly established Gersh and Bodian (1943) showed that the ultraviolet absorption spectrum of Nissl substance was identical to that of nucleoproteins and, further, that both the ultraviolet absorption and the characteristic tinctorial properties were destroyed by digestion in a solution of crystalline ribonuclease Thus, in nerve cells there is also a correspondence between nucleoprotein and protein synthesis In the intact nerve cell, which is presumably inactive with respect to protein synthesis, the nucleoprotein is apparently aggregated into clumps During the process of regeneration, it may be presumed that synthesis of new protein to reconstitute the axon must occur In this phase there is a dispersal of the cellular nucleoprotein to be followed again by its segregation into clumps at the close of the regenerative process

Basophilia may also be associated with protein synthesis in certain of the glands of internal secretion While investigating the human placenta, Dempsey and Wislocki (1944) were struck by the observation that both the syncytial and cellular trophoblast were strongly basophilic in early pregnancy, whereas in later stages the amount of basophilic substance declined until at term only occasional cytotrophoblastic elements retained their basophilic property By recourse to digestion with crystalline ribonuclease, these authors were able to identify the basophilic substance as nucleoprotein The correspondence in the amounts of basophilia exhibited in the trophoblast and the amounts of chorionic gonadotropin excreted in the urine during the various stages of pregnancy prompted the suggestion that the basophilic nucleoprotein might be concerned in the synthesis of the hormone Moreover, because the pituitary hormones are chemically similar to those from the placenta, Dempsey and Wislocki (1944) sought and found ribonucleoprotein to be a prominent cytoplasmic feature of certain cells of the anterior pituitary gland

Another example of the correspondence between cytoplasmic basophilia and protein formation stems from a comparative study of the placental barrier (Wislocki and Dempsey, 1945) The glandular and surface epithelium of the uterus, just prior to and during gestation, is rich in basophilic substance, greatest in the uterine glands of the pregnant sow, intermediate in carnivores, and least in rodents and in pregnant women In the sow, the epitheliochorial placenta consists of a simple apposition of the chorion against the intact endometrium Consequently, all of the nutritive substances obtained by the fetus must either traverse or be formed and secreted by the uterine epithelium The abundance of the uterine glands in the sow and the great quantities of ribonucleoprotein which they contain suggest that the proteins available to the fetus reach it through the uterine milk which is secreted by these glands In the

human, on the contrary, the uterine glands are probably relatively unimportant in the nutrition of the embryo, since in this species the uterine lumen is separated from the fetal tissues by the decidua capsularis Nevertheless, at the time of implantation and for a period thereafter, the paraplacental and subplacental glands are rich in glycogen, lipoids and basophilic substance, suggesting a nutritive rôle of the glands toward the free blastocyst, and the basal peripheral trophoblast (trophoblastic shell and cell columns) for some time following implantation Rodents, as exemplified by the rat and mouse, occupy a similar position with respect to the availability of the uterine secretions for the nutrition of the fetal adnexa In these two species the inverted yolk sac, after the dissolution of the capsularis and the parietal wall of the yolk sac, is exposed to the contents of the uterine lumen The uterine glands of the rat and mouse, beginning with progestation and extending throughout gestation, have an appreciable complement of basophilia In the cat, however, the uterine glands are intermediate between the sow, on the one hand, and man and rodents on the other, being larger and more active than those of the latter and possessing much more basophilia In this series of animals the amount of basophilic substance is greatest in the uterine glands of the pregnant sow, intermediate in those of the cat, and least in rodents and pregnant women

The last example which we wish to cite concerns the presence of basophilic inclusions in hepatic cells These inclusions are easily demonstrable after staining with pyronin or with methylene blue Their protein nature was readily established (Pfuhl, 1932) and they were observed to increase in size and abundance after eating Despite their relation to alimentation, it was demonstrated that they could not represent albuminoid substances because of their solubility characteristics Recently, Davidson and Waymouth (1944) have demonstrated that these basophilic inclusions in hepatic cells are abolished after digestion in a solution of crystalline ribonuclease Their nucleoprotein nature and their variation during alimentation have been confirmed in this laboratory (Deane, unpublished data)

The examples cited above come from diverse morphological locations and concern widely different physiological states Yet they have in common the fact that cytoplasmic ribonucleoproteins are contained within cells which are actively engaged in the synthesis of some kind of protein substance It is tempting to speculate that since various kinds of proteins are elaborated by the different cells, the corresponding nucleoproteins must also be different either in chemical composition or in spatial configuration It is to be hoped that in the future more refined methods will permit us to put to critical test the assumption that special proteins must have special templates upon which they are fashioned

B *The relation between nucleoproteins and phosphatase* We have already had occasion in an earlier section to allude to the chemical data which implicate phosphatase in nucleoprotein and nucleotide metabolism However, the rôle of phosphatase is far from completely understood Since nucleotides are in themselves organic phosphates, it is reasonable to suspect that their degrada-

tion into nucleosides might be a function of phosphatase, and indeed it may easily be shown in test-tube experiments that nucleotides and nucleic acids may be enzymatically dephosphorylated by phosphatase. On the other hand, an equally reasonable assumption might be that phosphatase serves the purpose of providing phosphate ions from some other phosphorylated substrate for the synthesis of nucleotides However, whatever the nature of the relationship between nucleoproteins and phosphatase may be, enough histochemical observations are now available to indicate that these two compounds frequently occur together and to suggest that they may be coupled into some kind of a metabolic process

The dissolution of the Nissl substance during retrograde degeneration and its subsequent reconstitution after regeneration of the nerve fiber have already been mentioned Bodian and Mellors (1945) have described parallel changes in the concentration within the nerve cells of phosphatases which operate over a wide range of pH During chromatolysis marked increases in phosphatase occur, the amount accumulating in the center of the perikaryon being sufficient to account for the eccentric displacement of the nucleus which has long served as a reliable indicator of retrograde degeneration Later, following regeneration of the axon, the accumulated phosphatase disappears as the Nissl substance is reconstituted and the nucleus returns to its original central position

A sequence of events rather similar to that discussed above takes place in the human placenta during the course of gestation Dempsey and Wislocki (1945) have described the gradual disappearance of basophilic nucleoproteins from the syncytial trophoblast of the chorionic villi in the course of pregnancy and the gradual appearance of alkaline phosphatase As the syncytium becomes less basophilic and more acidophilic, parallel preparations reveal increased amounts of alkaline phosphatase

The two examples presented above demonstrate an inverse relationship between ribonucleoprotein and phosphatase Such examples are compatible with the hypothesis that phosphatase acts to degrade or destroy the nucleoprotein However, we shall illustrate in the next paragraph that this inverse relationship does not always obtain, and therefore that the presence of phosphatase does not necessarily preclude the simultaneous presence of ribonucleoproteins

The cytoplasm of osteoblasts stains intensely with basic dyes, as is readily demonstrable in undecalcified preparations of bone After decalcification the basophilic property of the osteoblasts is largely lost because nucleoproteins are readily hydrolyzed in solutions of strong mineral acids Thus the basophilic quality of osteoblasts is best appreciated in preparations of embryonic bones where decalcification before sectioning is unnecessary or at most need be minimal Quite unlike the nerve cell and placental syncytium described above, the osteoblast contains abundant alkaline phosphatase in addition to its striking complement of cytoplasmic nucleoprotein The presence of phosphatase in these cells is a fact familiar to many by both chemical and histochemical methods

The observations cited above illustrate that phosphatases can exist in quite

different relationships with nucleoproteins in different cellular locations The thought obtrudes itself that osteoblasts may represent a situation in which phosphatase is secreted by cells to become active in the intervening intercellular matrix of nucleoprotein If this interpretation be valid, the concentration would find explanation in the rapid production and secretion of the protein phosphatase Definitive conclusions on these points in question cannot be drawn, but it is to be hoped that future investigations will correlate the occurrence of these two compounds in other locations

C *The relation between glycogen and phosphatase* As a result of the intensive studies of carbohydrate metabolism which have been carried out during the past decade, the biochemical events leading to the formation of glycogen are now fairly well understood It is generally agreed that a phosphorylated intermediary, such as the Cori ester, is a necessary antecedent to glycogenesis Beginning with this intermediary, the formation of glycogen then proceeds by dephosphorylation of the hexose phosphate which condenses in the specific environment of the animal cell into glycogen The transformation of hexose phosphate into glycogen requires the activity of a phosphate-splitting enzyme, and of the various enzymes capable of such action, alkaline phosphatase has been most frequently invoked because of its ubiquitous occurrence and because of its demonstrated capacity to utilize the particular substrates which serve as intermediaries in carbohydrate metabolism

Such considerations as those in the preceding paragraph lead to the thought that phosphatase activity represents the penultimate stage in glycogenesis It follows that the enzyme should be demonstrable in a region supervening between the source of the sugar and the location in which glycogen is being deposited This hypothesis lends itself to experimental proof by correlated observations on the presence of phosphatase and the deposition of glycogen

A series of comparative observations on the occurrence of phosphatase and the deposition of glycogen has been presented by Wislocki and Dempsey (1945) In both the maternal and fetal portions of the placenta in several species of animals it is found that the distribution of these two substances is in good accord with the hypothesis as stated above Nevertheless, several interesting differences in the spatial organization of the compounds has been noted In the sow, glycogen occurs only in the fetal chorionic cells but is entirely lacking in the maternal endometrium In this species, alkaline phosphatase is confined to the maternal endothelium and to the apical borders of the chorion Thus, the transmission of sugar from the maternal to the fetal tissues involves its passage across two regions in which phosphatase is demonstrable Since glycogen deposition occurs immediately after its passage across the second region, the thought lies close at hand that here is the region of final dephosphorylation which precedes the polymerization of the glycogen molecule

Other variants of the anatomical distribution of phosphatase and glycogen occur in still other species, but these various manifestations also bear out the concept enunciated above In the human endometrium during early pregnancy, phosphatase occurs only in the capillary endothelium, whereas glycogen

is densely concentrated in the epithelium In the pregnant cat, on the other hand, the deeply situated uterine glands contain both glycogen and phosphatase, and in this case no other deposits of the enzyme occur in the vicinity Consequently it appears that, although phosphatase generally occurs between the capillary bed and the region in which glycogen is deposited, the anatomical region which contains the enzyme is by no means constant It is to be hoped that similar correlated observations in other regions of the body will become available

## SUMMARY

The present review describes a selected series of staining reactions which have been applied to tissue sections and which have provided information capable of chemical interpretation The point of view is expressed that useful chemical characterization of cells and various interstitial substances can often be accomplished by methods which are not highly specific Furthermore, by the comparison of several different lines of evidence, conclusions may frequently be drawn when a single approach would be inconclusive

Several reactions which are reasonably specific for particular compounds or groups of compounds are described Among these are reactions for lipoidal substances such as lipines, steroids and vitamin A, and several enzymatic reactions including those for oxidases and peroxidase, the dopa reaction and phosphatases. The relationships between the substances revealed by these reactions and various physiological states are briefly discussed

It is pointed out that a system of histological classification based upon histochemical observations will eventually prove useful Such a system, employing chemical criteria, will be more directly applicable to the analysis of functional phenomena than the presently used morphological system As examples of this type of approach to histological problems, the relationships of nucleoproteins to protein synthesis, of nucleoproteins to phosphatase activity, and of phosphatase to glycogenesis are discussed

## REFERENCES

ASPLUND J, U BORELL AND H HOLMGREN Ztschr f mikr anat Forsch 48: 478 1940
ASTWOOD E B, J SULLIVAN A BISSBL AND R TYSLOWITZ Endocrinol 32 210 1943
BABKIN B D Secretory mechanism of the digestive glands Hoeber New York 1944
BAKER J R. Quart J Micr Sci 85 1, 1944
BAUER, H Ztschr f Zellforsch 15 225, 1932
Ztschr f mikr-anat Forsch 33 143 1933
BENNETT H S Am. J Anat 67: 151 1940
BICHAT, M Cited from General anatomy applied to physiology and medicine 1801
Transl by George Hayward Richardson and Lord Boston 1822
BIERRY H AND B GOUZON C R. Acad Sci 202 686 1936
BLOCH, B Arch f Dermat 124 129 1917
BODIAN, D AND R. C MELLORS J Exper Med 81: 469, 1945
BOURNE, G Quart J Exper Physiol 32: 1, 1943
BRACHET J C R. Soc Biol 133: 88 1940

CASPERSSON, T Arch f exper Zellforsch 22 655, 1939
J Roy Micr Soc 60 8, 1940
CASPERSSON, T AND J SCHULTZ Proc Nat Acad Sci 26 501, 1940
CORNER, G W Science 100 270, 1944
COWDRY, E V Microscopic technique in biology and medicine Williams & Wilkins, Baltimore, 1943
DAVIDSON, J N AND C WAYMOUTH Proc Roy Soc Edinburgh 62 96, 1944
Biochem J 38 39, 1944
DEMPSEY, E W Endocrinol 34 27, 1944
DEMPSEY, E W AND D L BASSETT Endocrinol 33 384, 1943
DEMPSEY, E W AND G B WISLOCKI Endocrinol 35 409, 1944
Am J Anat 76 277, 1945
DEROBERTIS, E AND J M GONCALVES Endocrinol 36 245, 1945
DESCLIN, L C R Soc Biol 133 457, 1940
DIETRICH, A Verhandl d deutsch path Ges 14 263, 1910
DOUGHERTY, T F AND A WHITE Am J Anat 77 81, 1945
DUBOS, R J Science 85 549, 1937
The bacterial cell Harvard Univ Press, Cambridge, 1945
EHRICH, W E AND T N HARRIS Science 101 28, 1945
EHRLICH, P Das Sauerstoff-Bedürfniss des Organismus Hirschwald, Berlin, 1885
Die Med Woche 15, 1901
EMMEL, V M Anat Rec 91 39, 1945
EVERETT, J W Anat Rec 91 10, 1945
FEULGEN, R AND H ROSSENBECK Hoppe-Seyler's Ztschr f physiol Chemie 135 203, 1924
FEULGEN, R AND K VOIT Pflüger's Arch 206 389, 1924
FIESER, L F Chemistry of natural products related to phenanthrene 2nd ed Reinhold, New York, 1937
FISCHLER, F Zentrabl f allg Path u path Anat 15 413, 1904
FLEXNER, L B J Biol Chem 131 703, 1939
FLEXNER, L B AND R D STIEHLER J Biol Chem 126 619, 1938
GERSH, I Physiol Reviews 21 242, 1941
GERSH, I AND D BODIAN Biol Symposia 10 163, 1943
GOMORI, G Proc Soc Exper Biol and Med 42 23, 1939
J Cell and Comp Physiol 17 71, 1941a
Arch Path 32 189, 1941b
Proc Soc Exper Biol and Med 51 133, 1942
GOULD, B S J Biol Chem 156 365, 1944
GREENSTEIN, J P Advances in Protein Chemistry 1 210, 1944
HARRIS, H A Nature 130 996, 1932
HARRIS, T N, E GRIMM, E MERTENS AND W E EHRICH J Exper Med 81 73, 1945
HELD, H Arch f Anat 396, 1895
HEMPELMANN, L H, JR Anat Rec 78 197, 1940
HOLLANDE, A Bull Hist appliq à la physiol 1 422, 1924
HOLMES, O W Border lines of knowledge in some provinces of medical science An introductory lecture Boston, Ticknor and Fields, 1862
HOLMGREN, H Ztschr f mikr -anat Forsch 47 489, 1940
HOLMGREN, H AND O WILANDER Ztschr f mikr -anat Forsch 42 242, 1940
HOROWITZ, N H J Dent Res 21 519, 1942
JORPES, E, H HOLMGREN AND O WILANDER Ztschr f mikr anat Forsch 42 279, 1937
KABAT, E A AND J FURTH Am J Path 17 303, 1941
KASS, E H Science 101 337, 1945
KFILIN, D Ergebn Enzymforsch 2 239, 1933

KIRKMAN, H AND A. E SEVERINGHAUS Anat Rec 70: 413 1938
Anat Rec 70: 557 1938
Anat Rec 71 79, 1938
KUNITZ, M J Gen Physiol 24: 15, 1940
LIPPMAN, F Ann. Rev Biochem 12: 1 1943
LISON, L Bull d'histol appliq à la Physiol 9: 177, 1932
Arch Biol 46 599 1935
Histochemie animale Gauthier Villars, Paris, 1936
MCCALLA T M J Bact 40: 23 1940
Stain Technol 16: 27 1941a
J Bact 41: 775, 1941b
MCCALLA, T M. AND F E CLARK Stain Technol 16: 95 1941
MCCLUNG, C E Microscopical technique 2nd ed Hoeber, New York, 1937
MACKENZIE, C G AND J B MACKENZIE. Endocrinol 32 188, 1943
MANN, G Physiological histology Oxford Press, Oxford, 1902
MICHAELIS, L. Biol Bull 87: 155, 1944
MIRSKY, A E Advances in Enzymology 3: 1, 1943
MITCHELL, A J AND G B WISLOCKI Anat Rec 90 261 1944
PFUHL, W Handbuch d mikr Anat d *Menschen 5 235 1932*
POLLOCK, W F Anat Rec 84: 23, 1942
POPJÁK, G J Path and Bact 56 485 1944
POPPER H Physiol Reviews 24: 205, 1944
RALLI, E P AND I. GRAEF Endocrinol 32 1, 1943
REICHSTEIN, T AND C W SHOPPEE Vitamins and Hormones 1: 345, 1943
ROBISON, R. 1922 Cited from The significance of phosphoric esters in metabolism New York Univ Press, 1932
SALTER, W T The endocrine function of iodine Harvard Univ Press, Cambridge 1940
SARASON E L Arch Path 35 373, 1943
SCHACHNER, H , A. L FRANKLIN AND I L CHAIKOFF J Biol Chem. 151 191, 1943
SOBEL, A E AND H WERBIN Fed Proc 4 104 1945
STOWELL, R. E Stain Technol 20: 45, 1945
SUMNER, J B AND G F SOMERS Chemistry and methods of enzymes. Academic Press, New York, 1943
TAKAMATSU, H Trans Jap Path Soc 29 492, 1939
TOLSTOOUHOV, A. V Stain Technol 3 49 1928
UNNA K., C V RICHARDS AND W L SAMPSON J Nutrition 22: 553, 1941
VERNE J Ann de Physiol 5: 245, 1929
WEED, L. H. Carnegie Contrib to Embryol 5: 1, 1917
WEINER, P Ztschr f mikr -anat Forsch 13: 197, 1928
WESTERFELD, W W AND C LOWE J Biol Chem 145 463, 1942
WISLOCKI, G B AND H S BENNETT Am J Anat 73 335, 1943
WISLOCKI, G B AND E W DEMPSEY Am. J Anat 77: 365, 1945
WOLF, A , E A KABAT AND W NEWMAN Am J Path 19 423 1943

# THE CORONARY CIRCULATION

DONALD E GREGG[1, 2, 3]

*Department of Medicine, School of Medicine, Western Reserve University, Cleveland, Ohio*

As in any other vascular bed, the influences controlling coronary blood flow are comprised of both physical and chemical factors The flow varies directly with the size of the vascular bed, the pressure head and inversely with the back pressure at the end of the system and the viscous resistance encountered in transit through the entire bed Aortic blood pressure determines the head applied at the coronary orifices, right auricular pressure (and to a smaller extent pressure in the right ventricle) determines venous back pressure Viscous resistance to flow is determined by the viscosity of the blood and the bore of the blood vessels The latter may be altered by two factors passive compression and relaxation resulting from rhythmical myocardial contraction (extravascular support) and active vasomotor changes in the vessels induced by nervous and/or humoral influences upon their intrinsic muscles

In past studies of the determinants of coronary blood flow, the venous drainage systems of the two ventricles were considered to operate in fundamentally different ways, and local mechanical forces were believed to be more potent in regulating flow than the chemical or humoral influences There has been a tendency to stress results obtained in situations where, although certain flow determinants were easily and completely controlled, it was neither possible to gauge the physiological normalcy of the preparation nor to determine the influence of the uncontrollable variables Recent studies in the dog have led to a re-examination of certain beliefs inasmuch as several new anatomical and functional concepts have arisen concerning the coronary venous system, collaterals, the coronary flow response to drugs, to increased cardiac load, and to sympathetic nerve stimulation Such modification of our working knowledge of the coronary circulation has followed the introduction of new methods and instruments and new and revised experimental procedures The object of this communication is to consider the development of these and other concepts, and to discuss the respective merits and shortcomings of the various devices and preparations used to study the coronary circulation and the factors which alter coronary blood flow

*Brief of the Anatomical Structures* The right auricle and ventricle receive most of their blood supply from the right coronary artery, and the left auricle and ventricle are supplied largely from the left coronary artery However, adjacent portions of the two ventricles and the intraventricular septum are

[1] Now at the School of Medicine and Dentistry, The University of Rochester, Rochester, New York

[2] The author wishes to express his indebtedness and appreciation to Dr R E Shipley, a former collaborator now at the Lilly Laboratory for Clinical Research, City Hospital, Indianapolis, Indiana, who has taken time to review the manuscript and to write that portion dealing with cardiac nerves

[3] This article was made possible by a grant from the Commonwealth Fund

supplied in varying amounts by both coronary arteries The sinoauricular and auriculo-ventricular nodes are usually supplied by the right coronary artery, while the right and left bundle branches are supplied by both coronary arteries, the arborization systems receive their blood from the arteries supplying the myocardium in which they lie (1, 2)

Comparatively few studies have been made of the capillaries of the heart muscle because of the great difficulty of their injection Wearn (3), using an injection technique for the isolated beating heart, succeeded in injecting the entire capillary bed and observed that the capillaries are parallel to the muscle fibers and that each muscle fiber lies in direct contact with one or more capillaries. During normal growth a relatively constant capillary muscle fiber ratio is maintained from birth to maturity During cardiac hypertrophy the muscle fibers enlarge, but the capillaries do not multiply and hence the capillary supply per unit volume of heart muscle is reduced (4, 5)

The venous drainage systems of the myocardium are 1, the superficial or subepicardial which empties into the right auricle and consists of the anterior cardiac veins and the coronary sinus together with associated veins emptying near its mouth, and 2, the deeper system of veins which communicates directly with the chambers of the heart

The deeper system of veins (2, 6) is made up of the arterio-luminal vessels (arterial communications between a coronary artery and the heart chambers), the arterio-sinusoidal vessels (thin walled endothelial tubes of capillary structure connecting arterioles with the heart cavities) and Thebesian vessels (channels between the coronary veins or the distal ends of capillaries and the heart cavities) These channels are much more numerous on the right side of the heart

The superficial drainage system of coronary veins extends over the surfaces of both hearts The greater portion of the subepicardial surface of the left ventricle is traversed by many small venous branches which merge to form the larger coronary veins These converge to form the coronary sinus, which, with associated veins emptying near its mouth, constitute the major superficial drainage system of the left heart In the right heart (and particularly evident in the dog) the small venous branches merge to form the anterior cardiac veins, anatomically the lesser of the superficial venous systems (7) They constitute several good sized venous trunks which lie buried in the subepicardial fat occupying the sulcus between the right auricle and right ventricle and each empties separately and directly into the right auricle a few millimeters superior to the ventricular border of the tricuspid valve Other smaller veins are invariably present The anterior cardiac veins, although described by Grant (8), have been virtually ignored by physiologists

In addition, there are numerous anastomotic channels connecting the coronary veins of auricles and ventricles with each other, with Thebesian channels, and with extracardial veins draining into the superior and inferior venae cavae (9)

In the anatomical sense, the coronary arteries are not end arteries Small arterial communications normally exist between the right and left coronary

arteries, between branches of the same coronary artery, and between coronary and extracardial arteries The intra- and extracardial communications, while small, are numerous and collectively have a considerable diameter With appropriate stimuli both arterial and venous anastomoses may become greatly enlarged (9, 10, 11)

EXPERIMENTAL APPROACHES TO THE STUDY OF THE CORONARY CIRCULATION

The development of adequate preparations and methods and the overcoming of instrumental obstacles is an essential preliminary to the solution of the problems dealing with the circulation Such consideration should reveal in part the proper basis for the interpretation of past and future studies of the cardiovascular system

The direct quantitative determination of total coronary flow requires the measurement of flow in all of the coronary arteries or veins Flow measurements made in only one artery or vein may or may not indicate correctly the directional changes in total coronary blood flow In general, when experiments are designed to permit measurement of total inflow or outflow, the preparation usually becomes sufficiently removed from the normal as to limit the interpretation of results

*Blood Flow Measurements with Devices Requiring Cutting the Coronary Vessel*

Each device must be inserted between the severed ends of a coronary vessel The instruments may be conveniently divided into those which measure the mean rate of coronary flow and those which measure continuously the phasic changes in the rate of inflow or outflow From the phasic flow curves mean rate of flow can also be determined

*A Mean rate of flow* Among the simpler methods for determining mean rate of coronary flow is the use of a mechanical stromuhr or the perfusion of one or more coronary arteries at a constant pressure and temperature from a calibrated perfusion reservoir (12) In the former the perfusing pressure and blood are supplied from the aorta and in the latter from a chamber outside of the animal The venturimeter (13), orifice meter (14), and Pitot tube (15) are basically the same in operation and record the pressure drop between two points in the flowing stream The pressure difference, which is proportional to the flow, may be read from water or mercury manometers or may be optically recorded by a differential manometer The rotameter (16) has been extensively used to record mean rate of blood flow in both the coronary and peripheral circulation Basically, this consists of a tapered vertical tube within which the height of a metal "float" varies with the rate of blood flow For continuous optical recording of flow, a small rotameter (17, 18) has been developed which operates in conjunction with an electrical unit There is also one type of thermostromuhr (21) that permits optical registration of mean rate of flow in cannulated vessels Its operation is similar to that of other thermostromuhrs and will be considered with them later Mean rate of flow may also be measured by timing the passage of an air bubble through a glass tube of known length and volume (19, 20) Of these devices the rotameter, Pitot tube, and orifice meter permit continuous optical recording of the flow changes

*B Phasic changes in instantaneous rate of flow* For the measurement of phasic changes in blood flow the recording device is designed to have a relatively high natural frequency which permits optical recording of the rapid fluctuations in velocity of flow (flow pattern) This instrument records the instantaneous flow at the point of its insertion into the blood vessel The different kinds of instruments have been considered in a previous review (22) and hence will be only briefly presented here

Certain of the phasic flow meters require the coronary perfusing pressure to be artificially maintained at a constant level, the coronary vessel being perfused from a reservoir partially filled with blood under the desired pressure The movements of blood from reservoir to coronary artery (or in the case of a coronary vein, from vein to reservoir) may be indicated by recording the cooling of a heated platinum wire placed in the air in the mouth of the reservoir (23) This device is unable to record rapid changes in flow and is also unable to indicate periods of retrograde flow (back flow) The perfusion reservoir may be closed, blood put under the desired constant perfusing pressure, and a sensitive calibrated optical capsule used to record the slight drop in chamber pressure (24) The calibrated slope of the declining pressure curve gives the rate of blood flow and the total drop over a period of time represents total flow This instrument has an adequate frequency and records with no appreciable lag However, because of the maintenance of a constant perfusing pressure, the use of either method permits deductions only with reference to the factors regulating the resistance to coronary flow

Phasic changes in blood flow may also be determined by methods in which the coronary artery is perfused by the normally pulsating aortic pressure Optical recordings have been made of the movements of a globule of mercury (25) or toluene (26) interposed in the coronary arterial stream There is considerable lag inherent in both methods The strömborste (27), as yet used only in the carotid artery and the aorta, has a high frequency and should have a place in the studies of the coronary circulation This device consists of a quartz fiber mounted between two electrodes and enclosed in a glass cannula inserted between the cut ends of the blood vessel Changes in stream velocity cause movements of the fiber which vary the resistance between the electrodes, thereby causing a current change through the recording galvanometer Of the phasic flow meters, the orifice meter (14, 28) has perhaps been most highly developed and extensively used to measure phasic flow in peripheral vessels as well as in the coronary circulation It was applied to the study of coronary flow with the hope that by an analysis of part or all of the flow pattern, the effect upon blood flow caused by cyclic changes in intravascular volume associated with volume-elastic changes and extravascular compression might be separated from the effect of intrinsic vasomotor changes in the coronary vessels As will be indicated later, this has been only partially realized

All of the aforementioned devices have certain advantages and disadvantages, some have superior technical aspects For example, the rotameter, orifice meter, and strömborste permit continuous recording of flow changes All of

the instruments limit flow to a variable extent and have the disadvantage of requiring their insertion into the severed vessel of an operated and anesthetized dog to which anticoagulants have been given Such procedures must induce a variable and unknown degree of insult to the cardiovascular system and its associated nervous and metabolic mechanisms, hence, the interpretation of the experimental results in terms of occurrences within the normal animal is necessarily restricted

*Measurement of Blood Flow with Intact Coronary Vessel* These methods do not require severing of the coronary vessel and in most instances no anticoagulant is required In some cases flow measurements may be made in the unanesthetized state

The electromagnetic flow meter (29, 30) is used to measure mean or phasic rate of flow As yet it has not been used to measure coronary blood flow Compared to the orifice meter flow curves, the patterns recorded with this instrument are somewhat "damped " This device utilizes the principle that an EMF is induced in the blood stream as it flows through a magnetic field The method would appear to be adequate for the measurement of mean rate of flow

Flow through the coronary vessels as well as other vascular beds has been determined by several types of thermostromuhrs Most of these instruments record the temperature difference of two thermal junctions applied to an unopened vessel when the vessel and blood between them are heated by means of high frequency current (31) or in direct current (32, 33) Bennett, Sweet and Basset (21) have devised a thermostromuhr onto which the severed blood vessel is tied and through which the passage of blood cools a heated thermocouple mounted on the end of a spicule projecting into the lumen of the device Schmidt and Hendrix (34) first suspected that the thermostromuhr was subject to a number of possible sources of error Subsequent extensive tests of this type of instrument (21, 35, 36) made *in vivo* and *in vitro* have indicated that the galvanometric deflection varies not only with the rate of flow but with a variety of factors such as the artery used and its degree of stretch, the degree of angulation of the unit with respect to the artery, the presence of periods of "zero flow" or retrograde flow (back flow) in the flow pattern, the immediate environment as regards composition and movements of intra- and extravascular fluid near the artery, and finally the viscosity of the metered fluid

The introduction of error by the appearance of, or a change in, the amount of retrograde flow (back flow) in the flow pattern was found to be of particular importance since flow patterns (28, 37) of all arteries thus far recorded with the orifice meter either normally contained back flow or it could be induced by the administration of drugs or by other procedures The thermostromuhr is particularly sensitive to such changes in that when the actual intra-arterial mean rate of flow decreases and short periods of back flow appear in the flow pattern, the thermostromuhr indicates a large increase in flow (35) Although it would appear to be a very difficult task, it is hoped that ways and means will eventually be found to eliminate the gross errors inherent in this method, since it is the only method of practical design which may be used in unanesthetized animals

Machella (38) measured phasic coronary flow by recording the cooling of a heated wire threaded through an otherwise intact coronary artery The flow pattern is somewhat "damped" as compared to that recorded by the orifice meter and no back flow components are present On a theoretical basis alone the instrument would be incapable of discriminating between forward and back flow and would be particularly inaccurate at low velocities of flow (21)

Changes in phasic coronary flow have been predicted by the so-called differential pressure method (39, 40) More recently with the aid of the rotameter, orifice meter (41) and constant pressure meter (42) researches have demonstrated that such a method may not indicate the directional shifts in flow In other instances, directional changes may be correctly indicated, but the magnitude of the flow change is not even remotely represented While such a procedure has added to our knowledge concerning flow determinants it must be discarded as an index of coronary flow

EXPERIMENTAL PREPARATIONS Since the time of Starling much information has been gained from experimental preparations in which the coronary circulation has been studied with the heart in various degrees of separation from the rest of the animal These preparations include the heart-lung (43, 44, 45, 46), the isolated heart with artificial lung (47), and the fibrillating heart (48) In general, the experimental findings have been considered significant and applicable to the normal heart beating *in situ* The chief advantage in using such preparations is that the several variables such as peripheral resistance, arterial and venous pressures, and cardiac output can be more easily controlled and the coronary flow more easily measured It is, of course, justifiable to determine what *can* happen with the hope of ultimately learning more about what *does* happen in the intact animal However, any great hope of success for this approach seems incompatible with the practice of removing the heart from its normal environment, work, and physiological controls and substituting an artificial set of new, and, in many instances, highly abnormal conditions There are many reasons for believing that much of the great bulk of work with these preparations has only a very limited significance and has yielded results rather far removed from the normal occurrences The apparent confidence that artificial regulation of the mechanical or tangible variables implies a well controlled (although abnormal) experiment is not warranted Important unrecognized changes can and do occur in the more intangible mechanisms of physiological function which invalidate many of the interpretations attempted When compared to studies with hearts beating *in situ* and under more nearly normal physiological conditions, the contour and values of the pressure curves, the coronary A V $O_2$ difference, the volume of coronary inflow, the distribution of coronary outflow, and the effects of different variables upon it, such as changes in cardiac output, cardiac load and coronary vein occlusion, are grossly different

Final judgment as to the validity of many accepted conclusions based on such experiments must await extensive reinvestigation in which utilization is made of preparations with greater functional similarity to the normal heart In any event, the continued preferential use of such preparations does not seem justifiable when more nearly normal preparations are now available

Studies have been made with the exposed heart beating *in situ* in the anesthetized dog, in which the coronary vessels were perfused from the aorta, using many of the devices described previously This is a closer approach to the normal animal, involving less insult to the animal's own nervous, metabolic, and cardiodynamic mechanisms Some of the studies presumably indicate with reasonable exactness changes which occur in the normal animal Even so, the observations can be accorded only limited interpretation

The ultimate objective is the quantitative measurement of coronary flow and the study of its determinants in the normal, unanesthetized animal Unfortunately, no device has, as yet, been developed which is adequate for this purpose

METHODS FOR ESTIMATING DETERMINANTS OF BLOOD FLOW CHANGES The determinants of coronary flow were indicated to be the pressure head, viscous resistance encountered in transit through the bed, and the pressures in the heart cavities at the end of the flow circuits Changes in aortic or central coronary pressure, venous, auricular, and ventricular pressure can be readily determined by the use of appropriate pressure manometers (49, 50) Viscous resistance to flow is governed for the most part by the mean calibre of the vascular bed Changes in this, the gross vasomotor state of the bed, can be recognized under certain conditions by correlating, as a simple ratio, the mean flow value with the corresponding mean applied pressure value However, the application of such an index is limited and should be made with caution Such an index does not take into account viscosity effects which, in a dynamic system, may give rise to considerable variation in the relationship of mean pressure to mean rate of flow, for which appropriate *in vivo* correction curves are not obtainable (28) Nevertheless, the vasomotor state of a bed may be regarded as having changed when mean rate of flow and mean blood pressure undergo considerable change in opposite directions Only partial success has resulted from attempts to identify and estimate separately the changes in two important variables which control the calibre of the coronary vessels, namely, extravascular compression (support) and active vasomotor changes in the vessels induced by nervous and chemical influences upon their intrinsic muscles

The problem of determining the magnitude of extravascular support has been approached in different ways 1 It has been suggested that intramural pressure may be taken as a measure of extravascular compression Phasic pressure pulses recorded from within the myocardium of the left ventricle by means of imbedded fluid pockets or arterial segments (51, 52, 53), are greater in the deeper lying myocardium, generally exceed the aortic or ventricular pressure simultaneously recorded, and are believed (51, 52) to approximate the correct value for intramural pressure However, *in vivo* and *in vitro* tests have shown that the relationship of the recorded intramural pressure to aortic pressure varies greatly with the level of inflation of the segment or pocket and with movement or distortion of the segment, and that if a vessel segment is placed within the left ventricular cavity the pressure generated within it may be 2–4 times the intraventricular pressure Mechanical protection of the segment reduces but does not eliminate the discrepancies (53) While the use of pro-

tected segments may possibly indicate directional changes, accurate quantitative measurement of intramural pressure is not possible by such methods 2 The maximum pressure transmitted during systole from the myocardium into a coronary artery, peripheral to the point of temporary occlusion of that artery, has been recorded in many dynamic states and has been used as an index of extravascular compression (14) In such a system elevation of the intracoronary vessel pressure during systole is related not only to the external compressing force of the myocardial fibers, but to the relative fullness of the bed and the ease with which the trapped blood can run off peripherally through the capillaries (vasomotor state) In order that changes in extravascular compression may be accurately indicated it is necessary that the vasomotor state of the bed and its relative fullness remain unchanged Since it is not possible to control or predict the constancy of these variables the method is to be discarded

As yet unsolved is the problem of determining the relationship of blood flow to active vasomotor changes, irrespective of whether the effect on intrinsic muscles of the coronary vessels is mediated through the blood stream or is secondary to metabolic changes in the surrounding myocardium As mentioned earlier, one may be certain of a change in vasomotor state whenever mean rate of flow and mean central coronary pressure change considerably in opposite directions However, it has been claimed that by analysis of phasic inflow curves changes in vasomotor state and extravascular support can be determined Critical points on flow curves have been selected in late diastole and systole at which the rate of change of volume elastic and myocardial compression forces are presumed to be minimal (14) At the diastolic point extravascular forces are at a minimum and the rate of flow reflects the vasomotor state of the coronary bed, at the systolic point extravascular support is maximal and the flow is said to reflect the combined effect of myocardial compression and the existing vasomotor state (54, 55) At these points the ratio of rate of flow to the simultaneously existing aortic pressure is determined A shift in the diastolic ratio is taken to represent active constriction or dilatation of the coronary bed Changes in the extravascular compressing force are determined by comparing the systolic and diastolic ratios Such deductions require the necessary proof of the ability of an indirect analysis to indicate correctly the actual occurrences Some of the assumptions upon which the validity of the method must necessarily depend are that 1, a period exists in which, throughout most of the myocardium, contraction of the myocardial fibers on the lumina of the whole coronary bed is exerted essentially simultaneously, 2, rates of flow taken at the critical points represent flow through the myocardium, 3, rate of flow is linear in both systole and diastole with respect to pressure Until these are verified experimentally such a system of interpretation cannot be regarded as having a sound hydrodynamic basis

*Arterial and Venous Collaterals* In the study of collaterals the coronary arteries may be occluded abruptly or gradually For chronic experiments an artery may be tied off abruptly, but more gradual constriction of the lumen may reduce the incidence of ventricular fibrillation, minimize infarction, and aug

ment collateral development The use of adjustable mechanical or osmotic clamps or the application of tight-fitting bakelite rings or cellophane bands to the artery will ultimately lead to complete coronary occlusion (56, 57) Unfortunately, by none of these methods can the time of complete occlusion be known, nor can the per cent reduction in flow be predicted even if the extent of local reduction in vessel lumen were known As in other vessels, the effectiveness of a given localized constriction in reducing flow may be large or small and will vary in inverse relation to the peripheral resistance of the vascular bed and lumen area of the constricted segment and in direct relation to the axial length of the constricted area, flow velocity and blood viscosity (58)

No entirely adequate method is available to measure collateral coronary blood flow in acute or chronic experiments after occlusion of a coronary artery An indication of intercoronary collateral development can be demonstrated experimentally by measurement of retrograde flow from a severed main coronary branch In acute and chronic occlusion experiments, with otherwise normal hearts, the retrograde flow of arterial blood is at first small, but this increases within a few hours, becomes considerably greater within a few days, and approximates in days to weeks the preocclusion value for the normal rate of inflow to that branch (59) This process is slow compared to that found in other less vital body regions Also, following chronic occlusion of a coronary artery, the pressure curve recorded in the artery peripheral to the occlusion may approximate that in the central coronary artery, and gross evidence of development of interarterial collaterals can be shown roentgenographically with the injection of a radiopaque medium (60, 61) If intracardial sources of collateral blood supply are insufficient, extracardial anastomoses also become evident and sizable extracardial communications can be demonstrated Under the most favorable conditions, the major portion of the coronary arterial system can be occluded with minimal or no infarction in those dogs that survive (66)

Many dogs do not survive more gradual experimental coronary occlusion In addition to gross coronary insufficiency, it has been suggested that the ultimate cause of death, generally ventricular fibrillation, probably stems from small zones of myocardial anoxia which are highly irritable, and is not necessarily related to a failing heart or gross coronary flow inadequacy Hence, if small amounts of blood via collaterals could be made to enter potential "trigger zones," it might prevent ventricular fibrillation and eliminate scar tissue formation through muscle preservation Accordingly, attempts have been made to subsidize the natural ability of growing tissue to develop collaterals Application of various tissue grafts have been used to form extracardial communications, epicardial or pericardial inflammation has been produced to foster intercoronary and extracoronary communications through mechanical or chemical irritation (62), or by the induction of cardiac venous stasis (63, 64, 65) Opinion based upon physiological experiments is divided as to the functional rôle played by such channels (66, 67, 68, 69) In any event there is a considerable reduction in mortality with all of these procedures, but with venous occlusion no definite difference in the size of infarct can be demonstrated (63)

*Distribution of Coronary Outflow* Direct measurement of coronary venous

outflow in isolated hearts and heart-lung preparations has shown that the coronary sinus and associated superficial veins emptying near its mouth constitute the major venous drainage system of the left coronary artery In such preparations the coronary sinus flow is taken to represent roughly from 50–65 per cent of the total coronary inflow in different cardiodynamic states (46, 70) Since simple and accurate methods for measuring coronary inflow were not available, such measurement of sinus flow has been widely used (with the heart beating *in situ*) to indicate quantitative and directional changes in flow Much controversy has arisen over the validity of this method, but the arguments are largely supported by observations made with the heart-lung or isolated heart preparation (46, 71, 97, 48, 73) The fact that, with the heart beating *in situ*, coronary sinus flow increases with elevation of right ventricular pressure, has in the past been taken to mean that coronary venous outflow is merely redistributed between the coronary sinus and Thebesian vessels in favor of the coronary sinus (15) This is offered as an argument against the use of coronary sinus flow as an index of coronary inflow It is more probable that the augmented sinus flow is predominantly the result of the increased inflow in both right and left coronary arteries which occurs (but was not measured in these experiments), especially since Thebesian flow is much less than previously supposed Experiments showing that the inconstancy of the coronary sinus fraction applies also with the heart beating *in situ* have not been reported It is probable that a more consistent and reliable evaluation of coronary inflow coronary sinus outflow relationship could be made with a less artificially controlled preparation which has been permitted to retain a more nearly normal state of hemodynamics However, from a practical standpoint the issue would seem of little import since there are now available adequate and simple methods of directly measuring coronary inflow in a somewhat more reliable preparation

From the use of similar preparations (heart-lung and isolated heart) it has been claimed that the Thebesian vessels drain most of the right coronary blood into the right ventricular cavity (73), *i e*, the functioning of the drainage system of the right heart was presumed to be considerably different from that of the left heart (48, 73) It is significant that in such studies it was assumed that all coronary venous blood not collected in the coronary sinus and appearing in the right ventricle must have come largely if not entirely from the Thebesian vessels In a recent paper Katz *et al.* (74) reported a reinvestigation of the problem in which the coronary venous drainage into auricles and ventricles was separated by an instrument made to expand in umbrella like fashion at the level of the A-V valves A considerable drainage into the right ventricle was again found and regarded as evidence that "a significant right ventricular Thebesian drainage occurs—which is an important element in coronary drainage" It would seem extremely hazardous to attach particular physiological importance to this finding since 1, it was apparently not noted what effect, if any, the presence of the instrument had upon the outflow from the anterior cardiac veins, the mouths of which open just superior to the ring of the tricuspid valve, and 2, the experiments were performed by perfusion of serum-saline mixtures through the coro-

nary vessels of an excised, non-beating heart, which conditions do not retain a reasonable similarity to those of a living, beating heart, still exercising its normal function in the animal

Recent work has demonstrated that with the heart beating *in situ*, the venous drainage of the right coronary artery is largely by way of the superficial anterior cardiac veins to the right atrium, under both normal and altered dynamic states The functional importance of these veins had been virtually ignored by previous investigators Experimentally, the direct cannulation and measurement of flow in only the major anterior cardiac veins has permitted recovery of from 50 to 92 per cent of the blood entering the right coronary artery (In addition, a certain fraction of blood from the latter drains into the coronary sinus ) These percentages are considered minimal, since for technical reasons no attempts were made to measure the outflow from the smaller anterior cardiac veins, the right auricular veins, and the many small veins of the right ventricle emptying into the great cardiac vein, all of which constitute channels of exit for blood supplied to the right heart by the right coronary artery (7) These findings in the beating heart make completely untenable the conventionally accepted belief that nearly all or even most of the right coronary inflow drains by way of the Thebesians into the right heart Most of the coronary venous blood from each heart appears to drain by way of its own system of superficial veins It follows that the studies of cardiac venous channels, flow partitions, and Thebesian drainage, which have been made in the past without measurement of flow in the anterior cardiac veins should be subjected to a critical revaluation before their associated conclusions can be accepted

*Luminal Vessels and Their Function* Histological studies, including serial sections, reconstructions, dye injections and perfusion experiments of the isolated heart (2) have been used to demonstrate the presence and distribution of Thebesian and luminal vessels through which the coronary vessels communicate with the atrial and ventricular chambers of the heart These channels are much more abundant in the right ventricle As already indicated, experimental studies in the beating heart *in situ* suggest that these luminal vessels may function in only a limited fashion in the undisturbed normal heart and to an extent very much less than previously maintained Actually, although many experiments have been devised to determine if, when, in which direction, and to what extent blood flows through these channels, none has been sufficiently conclusive to justify more than a speculation as to whether they ever function in the normal heart beating *in situ* The critical objections to most of the experiments can be resolved into either 1, the false assumption that all coronary venous blood entering the right auricle and right ventricle exclusive of that entering from the coronary sinus, has its origin in the Thebesian vessels, thereby ignoring the considerable venous flow from the anterior cardiac veins, and/or 2, the establishment by artificial manipulation of decidedly abnormal pressure gradients between the coronary vessels and chambers of the heart for the purpose of demonstrating what are implied to be the normal physiological magnitude and directional changes in Thebesian flow

It is of interest to examine the possible rôle of the Thebesian and luminal vessels in hearts in which either the coronary arterial or cardiac venous system is insufficient It is conceivable that the luminal vessels could be important, particularly if they could serve as arterial channels from the left ventricle to the myocardium during left coronary artery occlusion or as venous channels for the whole myocardium in the presence of extensive superficial vein occlusion Known observations are conflicting and any conclusion is difficult Regarding the first situation, with temporary functional separation of one or all coronary arteries from the aorta, no blood flow from the ventricles into the coronary arteries or superficial venous system could be demonstrated and the hearts did not survive (9, 75) Essentially complete occlusion of the coronary arteries in humans has been found at autopsy (76) but the presence and extent of development of extracardial arterial collaterals was not recorded When dye is injected into the right ventricle in acute experiments, extensive capillary injection occurs if right ventricular pressure is artificially made to exceed left ventricular pressure However, the anterior cardiac veins were not excluded as a vascular portal of entry for the dye (77) Regarding the second situation with acute closure of all grossly visible anterior cardiac veins or the coronary sinus, no great reduction in right or left coronary artery flow is usually observed The heart, following the acute closure of both the coronary sinus and anterior cardiac veins, becomes exceedingly hemorrhagic and tends to become progressively weaker, although such hearts may survive up to two hours Dogs in which both superficial venous systems have been chronically occluded in a two-stage operation have survived for a period of months This is suggestive evidence that all venous drainage occurred by way of luminal vessels However, that significant drainage occurred through such a route could not be verified, since at postmortem examination these hearts exhibited numerous superficial cardiac veins of considerable size which were not previously evident, and several large extramyocardial venous anastomoses, the aggregate cross-section of which might well have been adequate for the venous drainage of the entire heart (9) Until such intra and extracardial arterial and venous collaterals which appear with coronary arterial or venous ligation have been successfully removed or otherwise excluded as flow channels, a statement as to the utilization of Thebesian channels in diseased hearts is not warranted

*Responses of Coronary Circulation to Augmented Load* Of particular interest has been the subject of the coronary flow response to changes in the working load of the right and left hearts In experiments with the heart-lung and comparable preparations (but excluding those in which the vagi are intact), elevation of right ventricular pressure by constriction of the pulmonary artery or elevation of left ventricular pressure by aortic constriction (coronary perfusing pressure kept constant) has been demonstrated to cause a reduction in blood flow to the myocardium of right and left hearts respectively (78, 79, 80) The flow decrease is attributed to the dominant effect of the direct mechanical inhibitive action of the increased vigor of the heart and/or the establishment of an unfavorable pressure gradient, especially for the right coronary artery drain-

age via the Thebesian channels into the right ventricle Such a response would seem to be one of poor economy and certainly would not be advantageous to the heart when under an increased load However, in recent studies with the heart beating *in situ* and under more nearly normal physiological conditions, with vagi and sympathetics intact or severed, directly measured right and left coronary inflow increased appreciably upon elevation of right or left ventricular pressure (81, 82) In both instances cardiac venous outflow in both the coronary sinus and the anterior cardiac veins increased greatly (83) Measurements of cardiac output and $O_2$ consumption in the presence of an augmented load have demonstrated an increase in work and metabolism of the respective ventricles Under these conditions and in consideration of the increased coronary flow, the two ventricles have at their disposal a compensatory means by which their blood supply can, at least in part, be adjusted to their work and metabolic requirements Although the exact mechanism for the flow increase could not be identified with certainty in these experiments, it seemed probable that the coronary dilatation arose from an increased local production of metabolites and/or the creation of local relative anoxia due to the increased oxygen utilization The sustained flow increase is taken to indicate a dominant influence of coronary dilatation over the mechanical flow inhibiting effect of an increased extravascular support As already indicated these results are diametrically opposed to those of other investigators using less normal physiological preparations

While an increase in coronary flow is the net response to increased cardiac work, it can be shown to be antagonized by a flow-limiting mechanism (81) If, in the presence of an adequately maintained central coronary pressure, the pulmonary artery constriction and release is abrupt, the sustained flow increase described above may be preceded and followed by a transient period of flow reduction and elevation respectively, whose beginnings are coincident with the time of sudden constriction and release of the pulmonary artery The abrupt changes in coronary flow coincident with the onset of elevation and reduction in ventricular pressure are regarded as indications of a temporary separation of the influence of change in extravascular compression upon coronary flow This conclusion is made with reasonable justification since the increase or decrease in mechanical compression of the coronary vessels should occur simultaneously with the increase or decrease in intraventricular tension, while the slower physiological metabolic and vasomotor responses must necessarily lag somewhat behind their respective exciting causes, *i e*, the sudden changes in cardiac work The initial decrease in flow can be attributed to the dominant influence of augmented extravascular mechanical compression of the coronary vessels The transient flow increase following abrupt lowering of intraventricular pressure is a rough index of the extent to which flow had previously been retarded by the augmentation of extravascular compression

If such experiments are intentionally prolonged (4–5 hrs ), the right coronary flow response to pulmonary artery constriction is reversed and ultimately a sustained decrease in flow will be observed followed by a return to the control

level upon release of the pulmonary artery Thus, in the latter stages of an experiment during which the condition of the heart *in situ* has progressively become far removed from normal, the flow reducing effect of increased extravascular support is dominant over whatever flow promoting mechanism the heart has retained. As measured under these conditions and by others using heart-lung preparations, a decrease in coronary flow cannot be regarded as the "normal" response to an increased cardiac load

In the foregoing it was indicated that the coronary flow response to an augmented resistance load is the same for right and left hearts In all types of heart preparations central coronary artery pressure elevation *per se* is an effective means of increasing left coronary inflow Hence, in those instances in which the aorta was constricted peripheral to the coronary ostia, the blood supply of the left heart was additionally increased by the elevated central coronary pressure For this reason the left heart will operate at an advantage over the right heart in its ability to function with an increase in resistance load

An increase in the venous return to the heart beating *in situ* augments the coronary flow only mildly when aortic pressure is unchanged and to a much greater extent when the aortic pressure is permitted to rise (41) Here, as with increased resistance loads, the increased coronary flow presumably results from augmentation of cardiac metabolism and its associated dilator mechanisms More recently Katz *et al* (45) have concluded that the coronary flow increases with augmented cardiac output The explanation advanced is that the coronary vessels are passively dilated and their resistance to flow decreased as a mechanical adjustment to increased cardiac work and energy expenditure. This concept, at present, lacks the necessary explanation of the physical mechanism by which an increased cardiac output without an increased aortic pressure can of itself cause passive mechanical dilatation of the coronary bed

*Flow Patterns* Phasic flow curves have been recorded with the orifice meter in the coronary arteries and most major peripheral arteries under a great variety of dynamic states It is desirable to know to what extent such curves may represent events in the coronary bed within the myocardial walls For the peripheral flow patterns a method of analysis and interpretation has been presented, by means of which the probable determinants of, and their interrelated influences upon, phasic rate of inflow were discussed (37, 84) The coronary flow patterns are more complex In experiments with anesthetized dogs, left coronary flow patterns show flow peaks in systole and early diastole separated by short periods of reduced rates of flow or back flow during the isometric contraction phase, late systole, or early diastole (14, 22, 85) The right coronary flow pattern is not grossly dissimilar (82) The effects on the flow pattern of changes in blood pressure, heart rate, resistance load, of cardiac nerve stimulation, valvular deficiencies, and vasomotor drugs are multiple and have been separately reported (22, 41, 54, 55, 82, 85, 86, 87, 88) Unfortunately, the analytical approach for peripheral patterns does not encompass all the factors which comprise the determinants of coronary flow patterns In this case a comparable analysis is not possible because venous outflow is markedly pulsatile

and the arterial flow pattern is greatly influenced by compression and relaxation of extrinsic origin (extravascular support) A method of analysis using critical points on the flow curve has already been considered It is believed that no adequate system of analysis is as yet available for coronary flow patterns

*Drugs* Drugs may be effective in changing the myocardial blood supply through alteration of the blood pressure and/or the vasomotor state of the normal and collateral coronary vascular channels Certain of the drugs in addition alter blood flow by modifying the vigor of cardiac contraction Through lack of adequate methods great confusion still exists as to the mechanism of drug action, and in most instances no definite statement can be made

Pitressin decreases coronary flow by all methods of study and constricts the coronary bed, *i e* , flow decreases in the presence of an increased central coronary pressure (55, 87) It is believed that this drug acts by direct effect on the coronary vessels, but a decrease in cardiac metabolism and vigor of contraction has not been ruled out as the cause of the flow decrease

The flow response to the xanthines and nitrites is similarly consistent by all methods of study (54, 87, 88) The conclusion is inescapable that this group of drugs exerts a dilating action on coronary vessels, *i e* , coronary flow increases in the presence of a normal or reduced central coronary pressure A similar flow response occurs with papaverine when injected either intra-arterially or intravenously in dogs (89) Whether these drugs directly affect the vessels or cause the flow alteration by inducing cardiac metabolic changes is not known

In most preparations including the heart beating *in situ* epinephrine increases coronary flow (55) There is also an associated increase in vigor of contraction and cardiac metabolism The flow increase may be regarded as the net effect which results from an augmented extravascular support, tending to reduce flow, a metabolic dilator effect tending to increase flow, and whatever direct dilating effect the epinephrine may have at the time on the coronary vessels It is difficult to determine the respective magnitude of each separate effect Regarding its dilating effect *per se* on the coronary vessels, evidence is conflicting Green *et al* (55) found that following injection of epinephrine into the left coronary artery, the coronary flow increased simultaneously with elevation of blood pressure and increased vigor of contraction, while Shipley and Kohlstaedt (89) indicate that this is preceded by a transient period of flow increase without change in blood pressure This initial flow augmentation might then indicate a direct dilator action on the coronary vessels although, even here, an early metabolic influence cannot be ruled out

*Nervous Influences* The control of the coronary circulation by parasympathetic and sympathetic nerves has been a subject of much study The nerve supply to the heart is abundant although the anatomical separation of the two systems is somewhat difficult, particularly within the heart Wollard (90) has reported the presence of nerves, the terminal filaments of which pass among the muscle fibers and within individual muscle cells, and Nonidez (72) has shown the presence of parasympathetic fibers ending in the myocardium of the auricles and ventricles These findings suggest the possibility of nervous control over

myocardial contraction The abundance of nerves found in proximity to or ending on the coronary vessels implies a probable vasomotor function.

For the most part the nervous influences on the coronary circulation have been studied by observing the coronary flow responses following electrical stimulation or severance of the nerves Although such procedures are not paralleled by normal occurrences in the animal, the observed responses are presumed to indicate the functions which the nerves are capable of exercising in the intact animal Further difficulty in interpretation results from the fact that the specific effects upon the heart muscle and on the coronary vascular system are experimentally inseparable and only the net effect may be observed Differences in methods and preparations are additional variables which may account for the discordant results of different investigators

The vagus and sympathetic nerves are reported to contain both dilator and constrictor fibers according to different investigations (cf reviews by Wiggers, and McDowall (91, 92)) In general the so-called inhibitor and constrictor fibers are said to predominate in the vagus while the dilator and accelerator fibers are found in the sympathetic nerves The evidence for the constrictor effects has usually been the observation that abolition of the parasympathetic pathways (by mechanical or chemical means) results in an augmentation of heart rate or coronary flow (43) The normal presence of a tonic constrictor and inhibitor action is therefore implied

Studies of action potentials and the effects of faradic stimulation of the sympathetic nerves to the heart indicate that they probably function in maintaining and augmenting the rate and vigor of the heart beat (Cf 85, 93, 94, 95 ) Although coronary flow increases with sympathetic stimulation, the work and metabolism of the heart are also increased to such a degree that the accompanying coronary dilatation could be attributed to the increased local production of metabolites (95)

Katz and Jochim (96) have used the fibrillating heart in order that changes in blood pressure, vigor of cardiac contraction, and other variables could be excluded and purely vasomotor responses observed Their results are not conclusive evidence for the existence of a direct sympathetic vasomotor influence inasmuch as an increase in vigor of fibrillary contraction and cardiac metabolism was not excluded as the primary response which could secondarily give rise to vascular dilatation

It would seem most difficult to establish and identify conclusively by experimental means the separate effects of nervous influences upon the myocardium and coronary vessels because the physiological functions of these structures are so intimately related that their individual responses may be secondarily modified, each by the other

## COMMENT

The cardiovascular organs form a highly specialized system in which the coronary circulation plays a singularly important, although indirect, rôle in maintaining the organism in a normal hemodynamic state This fact alone

has amply justified the numerous studies of the coronary circulation and should continue to stimulate many future investigations

In the present paper no attempt has been made to organize a comprehensive review of all work done in the field and many topics of limited academic interest have been omitted In addition, the scope of this paper does not permit the thorough consideration of individual topics which may be found in separate reviews On the other hand, more emphasis has been placed upon the significance of studies with respect to their importance in revealing physiological occurrences which may have a parallel in the normal animal or human

Because of technical difficulty and, more often, because of the impossibility of making direct and positive observations of the dynamic occurrences in the heart, the investigator must often accept the alternative of observing the responses to variables introduced in an experimental preparation which is considerably removed from the normal state Except for the anatomical studies the reported observations dealing with coronary flow, its minute volume, distribution and ultimate drainage, its response to drugs, nervous and humoral influences, to changes in aortic pressure, peripheral resistance, cardiac output, cardiac work and metabolism, all are significant only in that they necessarily apply to the preparation and conditions under which the observations were made All too often the results obtained with a decidedly abnormal preparation ultimately come to be regarded as events which can and do occur in the normal animal or man

Various concepts concerning the coronary circulation have frequently been revised as new and improved instruments and preparations have been developed Much of what has been reported in the past should be discarded as more accurate methods are used for making the same physiological studies Observations made in the open-chest, but otherwise intact dog, must certainly be as cautiously interpreted as those made with any other preparation However, with the use of accurate methods and instruments, and in experiments of short duration the uniformity of the results suggests that such a preparation may approach the normal state of the animal somewhat more closely than many other preparations that have been used Until better instruments and methods are devised and used in conjunction with preparations which are capable of normal physiological responses, our knowledge concerning the normal and abnormal functioning of the coronary circulation will be necessarily limited as well as unavoidably inexact

## REFERENCES

(1) Spalteholz, W Die Arterien der Herzwand Leipzig, S Hirzel, 1924
(2) Wearn, J T Chapter I Diseases of the coronary arteries and cardiac pain R L Levy, New York, Macmillan, 1936
(3) Wearn, J T J Exper Med 47 273, 1928
(4) Shipley, R A , L J Shipley and J T Wearn J Exper Med 65 29, 1937
(5) Wearn, J T Bull Johns Hopkins Hosp 68 363, 1941
(6) Wearn, J T , S R Mettier, T G Klumpp and L J Zschiesche Am Heart J 9 143, 1933-34

(7) Gregg, D E , R. E Shipley and T G Bidder. Am. J Physiol 129: 732, 1943
(8) Grant J C B A method of anatomy Williams & Wilkins, Baltimore, 1940, p 507
(9) Gregg, D E and R. E Shipley To be published
(10) Hudson, C L , A. R. Moritz and J T Wearn J Exper Med 56 919, 1932
(11) Beck, C S Blood, heart and circulation AAAS Pub no 13, 1940, p 133
(12) Katz, L. N , K. Jochim and A. Bohning Am J Physiol. 122 236, 1938
(13) Schmidt C F Am. J Physiol 84 202, 1928
(14) Gregg D E and H. D Green Am. J Physiol 130 114, 1940
(15) Johnson J R. and C J Wiggers Am. J Physiol 118: 38 1937
(16) Gregg, D E , R. E Shipley, R. W Eckstein, A. Rotta and J T Wearn Proc. Soc Exper Biol and Med 49: 287, 1942
(17) Shipley R. E and E C Crittenden, Jr. Proc Soc Exper Biol and Med 56 103, 1944.
(18) Crittenden, E C , Jr. and R E. Shipley Rev Scient. Instruments 15 343 1944
(19) Soskin, S , W S Priest and W J Schultz Am. J Physiol 108 107 1934
(20) Dumke P R. and C F Schmidt Am. J Physiol 138 421, 1943
(21) Bennett S H., W H Sweet and D L. Basset J Clin. Investigation 23: 200, 1944
(22) Gregg, D E Blood heart and circulation Am. Assn Adv Sci Publication no 13, 1940 p 81
(23) Rössler, R. and W Pascual. J Physiol 74 1 1932
(24) Green, H. D and D E Gregg Am J Physiol 130: 97, 1940
(25) Anrep G V , J C Davis and E Volhard J Physiol 73 405 1931
(26) Stehle, R. L and K L Melville J Pharmacol and Exper Therap 46: 471, 477, 1932
(27) Bergmann, G Ztschr f Biol. 98 536, 1938
(28) Shipley R. E. D E Gregg and E. F Schroeder. Am. J Physiol 138: 718, 1943
(29) Kolin A Proc Soc Exper Biol and Med 46: 235 1941
(30) Katz L N and K. Jochim Medical physics Year Book Publishers, Chicago Illinois 1944, p 377
(31) Rein, H. Ztschr f Biol 87 394 1928.
(32) Schmidt C F and A M. Walker Proc Soc Exper Biol and Med 33 346, 1935
(33) Baldes, E J and J F Herrick. Proc Soc Exper Biol and Med 37: 432 1937
(34) Schmidt C F and J P Hendrix. Proc Assn Res Nerv and Ment. Dis 18: 229 1937
(35) Gregg D E., W H Pritchard, R. W Eckstein R E. Shipley A Rotta, J Dingle, T W Steege and J T Wearn Am. J Physiol 138: 250, 1942
(36) Shipley, R. E., D E Gregg and J T Wearn Am. J Physiol. 136: 263, 1942
(37) Pritchard, W H., D E. Gregg R. E Shipley and A. S Weisberger. Am. J Physiol 138: 731, 1943
(38) Machella T E Am. J Physiol. 115: 632 1936
(39) Gregg D E H. D Green and C J Wiggers Am. J Physiol 112: 382, 1935
(40) Green, H D , D E Gregg and C J Wiggers Am J Physiol 112: 627, 1935
(41) Green, H. D and D E Gregg Am. J Physiol 130: 126, 1940
(42) Gregg D E and H. D Green Am. J Physiol 130 108, 1940
(43) Anrep, G V and H. N Segall. Heart 13: 239, 1926
(44) Peters, H. C and M. B Visscher. Am. Heart J 11: 273 1936
(45) Katz L. N , W Wise and K. Jochim Am. J Physiol 143 479, 1945
(46) Anrep, G V Lane Medical Lectures Studies in cardiovascular regulation. Stanford University, California, 1936
(47) Katz, L N and M. Mendlowitz Am. J Physiol 122: 262, 1936
(48) Katz L. N , K Jochim and W Weinstein Am. J Physiol 122: 252 1938
(49) Gregg D E. and D Dewald Am J Physiol. 124: 435 1938
(50) Hamilton, W F I. Brotman and G Brewer. Am. J Physiol 107: 414, 1934
(51) Johnson, J R. and J R. Di Palma. Am. J Physiol 125: 234, 1939

(52) JOHNSON, J R Fed Proc Am Physiol Soc 4 37, 1945
(53) GREGG, D E AND R W ECKSTEIN Am J Physiol **132** 781, 1941
(54) BOYER, N H AND H D GREEN Am Heart J **21** 199, 1941
(55) GREEN, H D, R WEGRIA AND N H BOYER J Pharmacol and Exper Therap **76** 378, 1942
(56) THORNTON, J J AND F R MAUTZ Am Heart J **19** 404, 1940
(57) PHELPS, K R Am Heart J **21** 664, 1941
(58) SHIPLEY, R E AND D E GREGG Am J Physiol **141** 289, 1944
(59) ECKSTEIN, R W, D E GREGG AND W H PRITCHARD Am J Physiol **132** 351, 1941
(60) GREGG, D E, J J THORNTON AND F R MAUTZ Am J Physiol **127** 161, 1939
(61) MAUTZ, F R AND C S BECK J Thoracic Surg **7** 113, 1937
(62) BECK, C S Ann Surg **118** 788, 1943
(63) BECK, C S AND A E MAKO Am Heart J **21** 767, 1941
(64) GROSS, L, L BLUM AND G SILVERMAN J Exper Med **65** 91, 1937
(65) THORNTON, J J AND D E GREGG Am J Physiol **128** 179, 1939
(66) BURCHELL, H B Arch Int Med **65** 240, 1940
(67) BURCHELL, H. B Blood, heart and circulation Am Assn Adv Science, Publication no 13, 1940, p 139
(68) ROBERTSON, H F Am Heart J **10** 533, 1935
(69) ROBERTSON, H F Surgery **9** 1, 1941
(70) MARKWALDER, J AND E H STARLING J Physiol **47** 275, 1914
(71) ANREP, G V, A BLALOCK AND M HAMMOUDA J Physiol **67** 87, 1929
(72) NONIDEZ, J F Am J Anat **65** 361, 1939
(73) MOE, G K AND M B VISSCHER Blood, heart and circulation Am Assn Adv Science, Publication no 13, 1940, p 100
(74) LENDRUM, B, B KONDO AND L N KATZ Am J Physiol **143** 243, 1945
(75) STELLA, G J Physiol **73** 36, 1931
(76) LEARY, T AND J T WEARN Am Heart J **5** 412, 1929–30
(77) ECKSTEIN, R W, J T ROBERTS, D E GREGG AND J T WEARN Am J Physiol **132** 648, 1941
(78) ANREP, G V AND H HAUSLER J Physiol **65** 357, 1928
(79) DAVIS, J C Am J Physiol **123** 50, 1938
(80) VISSCHER, M B J A M A **113** 987, 1939
(81) GREGG, D E AND R E SHIPLEY Am J Physiol **142** 44, 1944
(82) GREGG, D E, W H PRITCHARD, R E SHIPLEY AND J T WEARN Am J Physiol **139** 726, 1943
(83) GREGG, D E AND R E SHIPLEY To be published
(84) SHIPLEY, R E, D E GREGG AND E F SCHROEDER Am J Physiol **138** 718, 1943
(85) GREGG, D E AND R E SHIPLEY Am J Physiol **141** 382, 1944
(86) GREEN, H D AND R WEGRIA Am J Physiol **135** 271, 1942
(87) GREEN, H D Am Assn Adv Sci Publication no 13, 1940, p 105
(88) BOYER, N H J A M A **122** 306, 1943
(89) SHIPLEY, R E AND K G KOHLSTAEDT To be published
(90) WOOLARD, H H J Anat **60** 345, 1926A
(91) WIGGERS, C J Chapter II Diseases of the coronary arteries and cardiac pain R L Levy, New York, Macmillan, 1936
(92) McDOWALL, R J S Chapter VI. The control of the circulation of the blood Longmans, Green, New York City, 1938
(93) BRONK, D W, L K FERGUSON, R MARGARIA AND D Y SOLANDT Am J Physiol **117** 237, 1936
(94) BRONK, D W, L K FERGUSON AND D Y SOLANDT Proc Soc Exper Biol and Med **31** 579, 1934
(95) SHIPLEY, R E AND D E GREGG Am J Physiol **143** 396, 1945
(96) KATZ, L N AND K JOCHIM Am J Physiol **126** 395, 1939
(97) KATZ, L N, K. JOCHIM AND A BOHNING Am J Physiol **122** 236, 1938

# RECENT EXPERIMENTAL STUDIES ON LEUKEMIA

J FURTH

*The Department of Pathology, Cornell University Medical College, New York Hospital, New York, N Y*

Advancements in knowledge of virus disease and of cancer had thoroughly prepared the ground for leukemia research so that during the third decade of this century investigators in different parts of the world discovered independently that leukemia of mammals is transmissible The causation of chicken leukosis[1] by a filterable agent had already been known (24) and studies of avian leukosis disclosed numerous facts on the nature of the agents causing leukosis which proved to be variants of chicken tumor viruses However, as knowledge advanced a gap appeared between avian and mammalian leukemias and it seemed that the solution of major problems of human leukemia would come from studies of this disease in mammals in which leukemia appears to be neither induced nor transmitted by virus. Recent research with plants, protozoa and animals disclosed the existence of transmissible cytoplasmic agents determining the character of cells. Physiological and pathological changes in cells are now viewed together as alterations of the same intrinsic cellular mechanisms which determine the character of the cell (19, 47) Avian leukosis, which readily yields a cytoplasmic agent modifying cells, may yet become the object of fundamental research

The basic change in leukemia is an alteration in the cell with acquisition of properties of a cancer cell including partial or complete independence from factors regulating growth and differentiation of normal cells The ease of induction of this change (leukemogenesis), the development of relatively homozygous strains of mice in which this change seems inevitable, the isolation of lines of transmissible leukemic cells of different types, have laid the foundations of modern research on mammalian leukemia

Historical aspects of these developments and their earlier phases have been well reviewed (25, 86, 32, 96, 38, 33b, 88, 60b, 17), and this review surveys only researches of the last few years To introduce these, it seems desirable to recapitulate conclusions of earlier observations

RÉSUMÉ OF EARLIER WORK Leukemia of mammals can be transmitted by malignant (leukemic) blood cells which proliferate in any genetically compatible new host, retaining their essential character, and bringing about the death of the host

The type of leukemia is determined by the character of the leukemic cell, the site of malignant change and the resistance of the host. The same line of leukemic cells may form tumor-like masses (lymphosarcoma or myelosarcoma) or systemic disease with no tumors, depending on the portal of entry (site of origin), the degrees of ability to form tumors or invade the blood stream are inherent characteristics of malignant blood cells

[1] Term proposed by Ellermann to include all types of leukemia

Strains of mice have been developed by continued brother and sister matings in which the incidence of leukemia is fairly constant (high, medium or low) and is characteristic of the strain This incidence is determined by genetical factors, modified by such nongenetical influences as acquired through the foster mother, caloric intake, etc If powerful, hereditary or extrinsic factors alone seem capable of producing the disease

Avian leukemia does not behave as a contagious disease yet it can be transmitted by cell free agents Some agents produce leukemia, others sarcoma and still others may produce both Myelo- and erythroleukosis are easily transmissible and one agent usually produces both Only a few cases of lymphoid leukosis, which is one of the most common diseases in chickens, proved transmissible Under usual conditions of laboratory passages the range of activity of an agent does not change

What are the hereditary factors, what is the magnitude and character of maternal, hormonal, nutritional and other influences which determine the incidence of leukemia, what is the change that takes place when a normal cell turns leukemic, and given such cells what are the forces that influence their growth, were the major topics of recent investigations on leukemia of mammals What is the nature of the agent of avian leukosis, the cause of the common avian lymphomatosis associated with paralysis and to what extent can a virus be changed, were the major problems of avian leukosis recently investigated

*Nomenclature* The term leukemia or leukosis implies neoplasia of a blood cell (hemoblastosis) including both the aleukemic and leukemic types of the disease Lymphosarcoma is used to designate tumors formed by lymphocytes, myelosarcoma for those of myelocytes, these are regarded as types of leukemia The suggestion to name leukemic blood cells malignant lymphocytes or myelocytes found wide acceptance It should be considered whether a prefix "mal" for malignant or "neo" for neoplastic would not simplify nomenclature (e g, neolymphocyte) Leukemogenesis is the process resulting in the development of leukemia Leukemogens are agents producing the leukemic cell Bioassay for leukemia is the determination of the presence of malignant blood cells by the injection of the cell suspension into animals highly susceptible to the cells to be assayed, at an age when they are free from leukemia

THE GENESIS OF LEUKEMIA Many factors co-operate in the causation of neoplasms The ever present modifying factor is the genetic constitution of the host Nursing conditions, hormonal activity, nutritional state and other factors inherent to life are leukemogenic influences of varying intensity Furthermore, organisms are subject to accidental exposure to chemical, physical and other agents which may be powerful leukemogens These factors are interrelated, e g, different agents can cause the same nutritional deficiency Vitamins may affect neoplasms through indirect effect on endocrine organs (12a), one agent may enhance or depress the action of another

The study of these influences requires the use of several inbred strains of animals because of genetic differences in response to given stimuli, e g, the C3H mice are susceptible to the induction of leukemia with estrogens but not

to that with methylcholanthrene and the reverse is true for mice of the F strain (62d)

*Heredity* The familial occurrence of leukemia in animals has been shown to be due to genetic factors By continued brother and sister matings relatively homozygous strains of mice have been developed in which a high percentage of the animals die of spontaneous leukemia Non leukemic males and females of such strains, mated with each other, yield mice with the same liability to leukemia as leukemic males and females, indicating the significance of non hereditary factors even in mice with hereditary liability The fact that a certain number of mice of a highly inbred strain fails to develop leukemia may be explained in the opinion of R R. Gates (40) by the conceptions of penetrance or by Heisenberg's principle of indeterminancy An illustration of the latter is radium decay which proceeds at a fixed rate regardless of extrinsic conditions, although it cannot be predicted which atoms will break down at a given time So, in homozygous strains, only a certain number of mice will develop leukemia at a given age even if environmental conditions are identical (40)

Such high leukemia strains of mice are *a*, C57 of MacDowell with an expression of leukemia of about 90 per cent, *b*, our Ak strain with about 70 per cent, and c, Strong's F strain (62a) with about 50 per cent of leukemia These figures are approximate There is some variation in different batche of the same strain due to non genetic modifying factors (80a, 81b, 18) In our Ak mice the figures vary from about 60 to 80 per cent, they are usually low when intercurrent diseases are prevalent and high when they are absent

In hybrid mice between high and low leukemia strains not only is the incidence of leukemia lower than in the high leukemia strain but the onset of the illness is markedly delayed

In mice of the high leukemia strain spontaneous leukemia usually occurs when the mice are about 6 to 9 months of age When leukemic cells are introduced into immature mice they produce leukemia rapidly, hence, young animals are excellent objects for the identification of malignant blood cells, a test termed bioassay F1 hybrids between high and low leukemia strains are usually susceptible to grafts of leukemic cells arising in their parents even though they are but slightly or moderately liable to develop spontaneous leukemia Bioassays indicate that the leukemic cells are not present at birth in normal mice of a genetically susceptible strain and that the malignant change precedes shortly the onset of leukemia (84a, 98)

Cohnheim's theory that neoplasms originate in cells misplaced during embryonal life or in cells remaining in their normal sites but so constituted as to become malignant after a given age, or in cells that are perhaps malignant at birth but are restrained until conditions favor their multiplication, has been revived by Fischer He investigated the genesis of carcinoma of the breast, a neoplasm that arises in an organ undeveloped at immature age The sequence of changes is particularly conspicuous in male animals, first, the development of breast tissue followed by hyperplasia, which under intensified physiological stimulation may go on to neoplasia at multiple sites, particularly when the "milk agent"

is carried by the mouse Thus cancer may arise in cells that are non-existent before the stimulus producing it is applied

The tools of genetic studies are the inbred strains The mode of inheritance of leukemia varies with different strains and the negative influence from the low leukemia strain is as important as the positive influence from the high leukemia strain A high leukemia strain crossed with one low leukemia strain (Ak x Rf) may yield few leukemias while the same high leukemia strain crossed with another low leukemia strain (Ak x C3H) will yield many leukemias (37) The genetic influence of the low leukemia strain Rf is so strong that in the first back cross generation to this strain the incidence of leukemia is almost as low as in the low leukemia strain The incidence of leukemia in F1 hybrids of the F strain with several low leukemia strains is about as high as in the F strain (60)

Mercier crossed a high leukemia strain (with an expression of leukemia of 49 2 per cent) to a low leukemia strain and none of the F1 hybrids developed leukemia, leading to a conclusion identical with that of Slye (103), that leukemia is inherited as a recessive character In the F2 hybrids leukemia reappeared However it is possible that the same high leukemia strain crossed to another low leukemia strain (e g , C3H) would yield a high percentage of leukemic mice Indeed, it is possible that the hybrids of two low leukemia strains will yield many leukemias What matters is the combination of genes, and what this will yield in terms of leukemia only a test can tell

Surveying the reported data on two sets of crosses MacDowell et al conclude that the proportion of leukemias is a mathematical function of the total heredity from the high leukemia strain In crosses C58 x StoLi, the function appeared to be directly arithmetical (81b) In crosses of Ak x Rf the function was semi-logarithmic with the logarithm of the percentage of leukemias varying with the proportion of high leukemia heredity (18) This difference may, in their opinion, be due to extrinsic variables and with the number of such variables unknown such experiments do not define the number of genes responsible for the parental influence (81b) The mere appearance of a neoplasm in an animal or the frequency of neoplasms in an inbred strain is no indication of intrinsic tendencies The unique characteristic of genes is their assortment in cross breedings (82)

The complexity of the genetic basis of susceptibility to leukemia is illustrated by the second back cross test of MacDowell et al (81b) for determiners of spontaneous leukemia In the F1 generation, theoretically all mice are genetically alike but in the back cross generation the number of different genotypes increases with the number of genes involved in the character studied If one or two major genes were involved in susceptibility to spontaneous leukemia, then there would be only two or four different genotypes with respect to leukemia among the males from the first backcross generation The data indicate that the males of the first backcross generation were of many genotypes and this is in agreement with the hypothesis that spontaneous leukemia depends for its development upon many genetic factors The differences in the results of various workers can be explained by genic differences among the strains used

and by the many unknown extrinsic variables Environmental factors modify the expression of genetic tendencies and different types of leukemia may have different genetic bases (18)

An admirable analysis of the numerous variables mother's age, strain of nurse, sex, hair color and longevity in the same mating combinations, is contained in the recent paper of MacDowell et al (81b) Longevity itself is influenced by the genetic constitution of the father, by the parturition age of the mother, by the strain of the nursing female and by the sex, each influence acting differently (81b)

The evidence that genes are a strong influence on the incidence of leukemia is conclusive but it is unknown how many genes are involved and how they exert this effect Experiments to be described below suggest that the leukemic cell is not inherited as such and that the malignant transformation takes place in certain loci during life If one considers that differences in response to leukemogenic agents often do not parallel differences in liability to spontaneous leukemia and that susceptibility to one agent does not indicate susceptibility to another agent it becomes clear that we are far from knowing the rôle of heredity to spontaneous leukemia

Data available on the heredity to leukemia in man are meagre and inconclusive The heterozygocity of the human population and strong influence from non leukemic parents may mask genetic tendencies The occasional appearance of this disease in infancy certainly indicates a congenital if not a genetical factor

*A maternal influence* in the occurrence of leukemia is indicated by the incidence of leukemia in reciprocal matings

| | MATING COMBINATION H/L | | |
|---|---|---|---|
| | C3H/StoLI | Ak/Bl | Ak/C3H |
| | Author | | |
| | MacDowell et al. | Cole et al. | Furth et al. |
| High leukemia strain | 89 6 | 69 3 | 58 |
| H ♀ x L ♂ | 61 9 | 21 9 | 50 |
| L ♀ x H ♂ | 42 5 | 11 6 | 34 |

*H = high leukemia strain
L = low leukemia strain

The existence of a maternal influence led to a search for a milk factor similar to that of Bittner's milk agent Foster nursing by low leukemia dams reduces the incidence of leukemia in the high leukemia strain to about one-half but mice of a low leukemia strain nursed with high leukemia strain dams do not develop leukemia This lowering of the incidence of leukemia cannot be explained by failure of the mice to reach the leukemia age (2, 33c) since mice fostered by low leukemia strain mothers live on the average longer than those fostered by their own mothers If C3H dams, which carry a strong milk factor,

are used for foster nursing high leukemia strain mice, the latter will develop mammary gland carcinoma while the C3H mice will not develop leukemia The incidence of leukemia in F mice fostered by low leukemia strain mice was lower (28 per cent at 500 days) than in unfostered mice (38 per cent at 500 days), the reciprocal foster nursing did not produce leukemia in the low leukemia strain mice (62c)

Thus nursing influence for leukemia, unlike that for mammary gland carcinoma, is not transmitted to offspring yet it may be related to the virus-like milk factor for mammary gland carcinoma If this agent is conceived to be a virus, it is subject to reactive forces of the host which may mask its existence as is the case with the virus of Shope (cf Rous)

The superiority of a strain ("B alb") in nursing has been demonstrated by MacDowell et al (cf 81b) in terms of body weight, tail length and lengthening of life This effect is greater in males and is gradually lost during nine months In MacDowell's experiment (81b) the nurse strain influence appears to be due primarily to the accidental presence of certain sex limited non-leukemic conditions shortening life This does not seem likely in our experiments (33c)

The parturition age of the mother is a strong modifier of genetic factors The increasing parturition age of the mother progressively delays the appearance of leukemia (81b) Thus, youngest mothers reveal more accurately the potential leukemics The nature of this phenomenon is not known Chemical and physiological changes occur with increasing age The influence might be imparted to the young through the egg cytoplasm or through the placental circulation, or through the first milk (81b) It may be related to the nursing influence discussed The placenta is no barrier to maternal hormones or to agents of the size of viruses—though the milk "virus" does not seem to pass through the placenta Immunization of the mother against substances in the fetus is intensified during subsequent pregnancies as indicated by observation on the Rh factor Is a similar neutralization phenomenon operative against the hypothetical agent producing leukemia or do the factors causing leukemia begin to operate during embryonal life and decline in intensity with advancing age of the mother?

The observations described indicate that susceptibility to leukemia is at its peak when the high leukemia strain mouse is nursed by its mother and she is young when she nurses

*Sex and sex hormones* A sex factor is operative in most strains of mice Data tabulated by Cole (18) show that in most strains females have a higher incidence of leukemia than males It is remarkable that in man the reverse is true, the ratio being about 2 1 in favor of males

This sex difference is small in mice, and may not be statistically significant, as in the recent experiments of MacDowell et al (81b) These workers explain the higher incidence of leukemia among females in their experiments by the greater longevity of the females They suppose that females resist certain factors that shorten the life of males and in so doing permit more of the potential leukemics to develop leukemia (81b) Mortality curves in other strains indi-

cate however that most males survive their leukemic sisters In view of the unquestionable leukemogenic effects of estrogens, the greater incidence of leukemia among the females should be considered to be due in part at least to the female sex hormone But thus far, experiments made to verify this supposition have not yielded uniform results

In the high leukemia strain (Ak) the incidence of this disease was lowered from 74 per cent to 45 per cent by ovariectomy performed at 23 to 56 days of age. Among males subjected to orchidectomy at 20 to 56 days the incidence was 60 per cent as compared with 52 per cent among the controls (84) In Gardner's strains in which the incidence of leukemia in females is not higher than in males, the ovariectomy had no significant effect (41) Similarly in the F strain of Strong (60a) the incidence of leukemia is about the same in both sexes and gonadectomy did not significantly alter these figures

In a strain of Lynch (R.I.L ) the incidence of leukemia in the females was 88 4 per cent, in males 53 5 per cent Castration in males raised this figure to 97 per cent while the administration of testosterone propionate to castrates lowered the latter figure to 58.3 per cent Ovariectomy had no significant effect on the incidence of leukemia (78b) Removal of the gonads in these experiments was performed at about weaning age or shortly thereafter Murphy suggests that the sex difference in susceptibility to spontaneous leukemia is due to an inhibitory effect by the male sex hormone rather than to a stimulation by the ovarian secretion But the leukemogenic effect of estrogenic hormones is amply proven by the experiments of others The interpretation of all of these experiments is rendered difficult by the unknown degree of compensation of the adrenals for the loss of ovarian sex hormones In man, relapses after castration at old age for carcinoma of the prostate are attributed to the compensatory production of the male sex hormone Castration at one day of age leads to hyperplasia of adrenals and occasional tumor formation with excessive sex hormone production It would be of interest to know the effect of castration at various ages on the incidence of leukemia and the alteration of the hormonal balance by castration

The leukemogenic action of sex hormones is indicated by the experiments of Gardner et al (41, 42), Lacassagne and Bischoff et al and of others (cf 42) The well controlled experiments of Gardner indicate that in certain strains estrogens are as powerful leukemogens as any known agent (41) Other strains of mice (e g , C57 of Gardner) are entirely refractory to the leukemogenic action of estrogens even though other organs of the same mice are highly responsive to them The leukemogenic action of the chemicals tested roughly parallels their estrogenic power and can be neutralized by androgenic hormone Testosterone does not influence the occurrence of leukemia in a low leukemia strain of mice (41) Discontinuation of estrogens after 2½ to 3 months resulted in the production of more lymphoid neoplasms than continuous exposure, presumably because the animals lived longer Thus, a short but intense treatment is capable of bringing about irreversible changes leading to leukemia. It will be of great interest to learn how brief and how intense this treatment has to be to produce this change when other conditions are set for it

Susceptibility to estrogens is under genetic control of the host (41) In hybrid mice with known susceptibility to estrogens the leukemogenic action of estrogens varied greatly Hybrid mice born of parents of two susceptible strains had the highest incidence of leukemia following estrogen treatment, none occurring in the untreated controls When both parents were of the resistant strain the hybrids had in one combination a greater, and in another combination a lesser susceptibility to estrogens than the parents (41)

How do estrogens produce leukemia? Their primary effect is an atrophy of the lymphoid organs including the thymus and bony proliferation with replacement of the marrow The leukemias produced are of the lymphoid type and arise in the atrophic organs, frequently in the mediastinum, and are indistinguishable from spontaneous leukemias of the same type Mediation by the adrenals is not required Estrogens act on lymphoid organs even in the absence of adrenals (101) X-rays are also leukemogenic and the primary change is likewise that of depression of hemopoiesis Neoplasms often arise in atrophic organs, but the factors which push focal areas of regeneration over to the neoplastic side remain a mystery

*Thymus* It has been noted that the thymus is a frequent primary site of leukemia in a strain of mice (Ak) with high hereditary liability to spontaneous leukemia Removal of this organ in this strain at 31 to 71 days of age resulted in a reduction of the incidence of spontaneous leukemia from 77 to 8 per cent in females and from 61 to 11 per cent in males (84) Removal of the spleen at comparable ages had no effect on the incidence of spontaneous leukemia The life span of thymectomized Ak mice is on the average about one half longer than in normal Ak mice and this is probably due to prevention of death from leukemia

The presence of the thymus is important in leukemogenesis in at least this strain of mice Moderate doses of methylcholanthrene produce leukemia sooner and in larger numbers of animals whose thymus is present (33e) In this strain the thymus is frequently the primary site of the disease and it may be supposed that its lymphoid cells are especially liable to a malignant change.

Investigations on the possible mechanisms by which the incidence of leukemia is lowered by thymectomy disclosed the following *a*, the effect of thymectomy is not inherited, the incidence of leukemia being as high in the offspring of thymectomized mice as in their normal grandparents (33e), *b*, thymectomized mice are as susceptible to the growth of grafted leukemic cells as normal mice (33e), *c*, removal of the thymus reduces susceptibility to the leukemogenic action of methylcholanthrene but not to the carcinogenic action of this hydrocarbon, *d*, administration of thymus from mice of a low leukemia strain to thymectomized mice over a period of four months does not seem to restore susceptibility to leukemia (33e) However, it is possible that the "substitution therapy" was not adequate

These observations suggest that in mice of the high leukemia strain Ak, potentially neoplastic blood cells reside in the thymus and that removal of this organ causes reduction in the incidence of leukemia not by virtue of an endocrine factor but by removal of potentially malignant cells

If the above assumption is correct, it may be expected that the Ak thymuses retain malignant potentialities after transplantation in a manner as limbs of creeper fowl embryos develop as achondroplastic when grafted on normal embryos It seems desirable to remove the thymus in strains of mice in which leukemia is myeloid or lymphoid but not thymic in origin and to study the effect of thymectomy on other neoplasms

Accidental involution of the thymus often accompanies malnutrition, intoxication or disease The experiments described show how these can modify an expression of genetic susceptibility to lymphoid leukemia

*Adrenal.* The reciprocal relationship between thymus and adrenal cortex is well known (cf 89, 22a, 22b) It follows that hyperplasia, hypoplasia or dysfunction of the adrenal cortex may modify the incidence of spontaneous leukemia or the response to a leukemogen However, there are no experimental studies on record on such an effect The conspicuous effect of adrenal cortical hormones on the growth of leukemic cells is discussed below

*Nutritional influence* Tannenbaum (108a, 108b) has presented statistical and experimental evidence indicating that restriction of caloric intake reduces the incidence of neoplasms, in man and animals Weight loss alone, what ever its cause, can be regarded as exerting an inhibiting influence on the incidence of neoplastic diseases

The effect of reduction in food intake on the genesis of spontaneous leukemia is indicated by the experiments of Saxton et al (98) In mice of a high leukemia strain receiving limited amounts of a qualitatively adequate diet, the incidence of leukemia was 10 per cent as compared to 65 per cent in normally fed controls Bioassays at 9 to 12 months indicated the absence of malignant lymphocytes in the underfed mice, and disclosed their presence at 14, 16 and 17 months. In normally fed mice of the same strain, malignant cells appeared at 7 to 13 months. These experiments indicate that *a*, malignant lymphoid cells are absent in young normal mice of the high leukemia strain Ak, *b*, the malignant transformation takes place shortly before leukemia becomes manifest, and *c*, this transformation is delayed but not entirely prevented by underfeeding (98)

It is possible that the effects here described are due to some specific nutritional deficiency due to the overall reduction of food intake although none was noted Underdevelopment of thymus and lymphoid tissue might possibly explain a lowering of the incidence of leukemias but they can hardly account for the inhibition of other neoplasms by underfeeding Thymectomy produces a marked reduction in the incidence of leukemia without causing weight loss of the experimental animals (33e)

In mice "painted" with methylcholanthrene, a 50 per cent caloric restriction reduced the incidence of leukemia from 96.2 per cent to 35 per cent and increased the mean latent period from 73 to 237 days (115)

Lack of cystine in the diet also reduced the incidence of leukemia in mice "painted' with methylcholanthrene The addition of gelatine to a low cystine diet raised the incidence of induced leukemia from 10 to 32 per cent and this effect is explained by the presence of a small amount of cystine in gelatine (116)

There are no data on the effect of low cystine diet on the incidence of spo taneous leukemia

Nutritional deficiency has been known to cause involution of the thymu Deficiency of riboflavin, pantothenic acid and thiamin or pyridoxin can cau depression of the weight of the thymus (106) Depression of lymphoid orga usually accompanies atrophy of the thymus and any agent which brings abo atrophy of this organ might influence the incidence of lymphoid leukemia

*Chemical leukemogenesis* Earlier experiments suggested that tar and ca cinogenic hydrocarbons are also leukemogenic (10, 25) Morton and Mid described a readily reproducible method to induce leukemia with such an agen They "painted" repeatedly a solution of methylcholanthrene on the skin mice at different sites so as to minimize the development of local tumors Th procedure, they believed, hastened the onset of leukemia in genetically su ceptible strains of mice However, some low leukemia strains proved equall susceptible to this hydrocarbon

The quantity of methylcholanthrene sufficient to produce leukemia is smal Six "paintings" with a 0 5 per cent solution of methylcholanthrene in benz applied twice weekly over an area of a few centimeters has raised the leukem incidence and hastened its onset Two paintings had little effect, eight painting were better than six but more intense treatment did not raise the leukem incidence (33d)

The efficiency of a hydrocarbon in hastening the appearance of leukemia i the high leukemia strain F bears a direct relation to the potency of the hydro carbon to induce breast tumors (62d)

There are differences in susceptibility to leukemogens among different strain of mice (61) A strain resistant to the leukemogenic action of x-rays may b susceptible to that of methylcholanthrene and vice versa (60b)

The leukemias produced by hydrocarbons are usually of the lymphoid type and "atypical" (see below) There is no clear demonstration of the ability of these substances to induce myeloid and monocytic leukemias although there are experiments on record suggesting such potentialities (33b)

In most of these experiments benzol has been used as solvent Benzol itself is leukemogenic (cf 60b) but not under the conditions tested Solvents may have a pro or anti-carcinogenic action, though alone they do not produce new growth (12b)

The leukemogenic action of benzol is suggested by human observations (32) the few experimental studies on record suggest that it is a weak leukemoge (cf 25, 60b) The work of Büngeler et al (60b) suggesting that indol is leu kemogenic remained unconfirmed

2-amino and 2 acetoaminofluorene when fed to rats produces neoplasms i different organs (118) including leukemias Bielschowsky (a) noted four leu kemias in 104 rats of a non-leukemic strain fed on this compound but most o these rats had other neoplasms affecting the liver, mammary gland, etc , an these may have masked the leukemogenic potentialities of this compound

ter studies of Bielschowsky (b) suggest that as a coleukemogen this compound ght be unusually effective

*Physical leukemogens* X rays have long been known to cause leukemia in an (cf 32, 52) and they produce it readily in mice According to a recent rvey the incidence of leukemia in radiologists for a fifteen year period is ten nes greater than in physicians who are not radiologists (70)

There is little known about the factors of irradiation responsible for the velopment of leukemia The doses which have this effect are large enough to stroy the bone marrow Regeneration follows atrophy but the stigmata of assive irradiation persist in experimental animals Hemopoietic tissues once assively irradiated may never be normal The spleen shrinks, connective sue increases in amount and the organ is laden with hemosiderin filled macro- ages Large areas of atrophy or necrosis have been a common autopsy finding the femoral marrow of old mice that had been given about 350r at approxi ately six weeks of age (33d) Some cells among those newly formed acquire e property of unrestrained growth Both myeloid and lymphoid leukemias e produced by x rays Henshaw (b) has been most successful in inducing ukemia with x rays He has given doses of 200r for 3 to 4 month old mice, peated at four week intervals for twenty weeks (total of 1200r) and thereby oduced leukemia in 30 per cent of C57 mice Blood counts showed in general, ukopenia, but in some animals, recovery was followed by marked leukocytosis th leukemic infiltration at death Leukemia was predominantly of the imitive cell type and death occurred usually between the seventh and twelfth onths. However, the incidence of leukemia among his controls was also gh (7 per cent) There is no study on record which would give quantitative formation on the relation of the dose and quality of x rays and mode of applica- n, to the incidence of leukemia Neither are the meager data on strain fferences in susceptibility to x rays convincing It appears from the survey the literature that x rays are weak leukemogens and act after a long incuba- n period In a small series, irradiation of F1 hybrids between a high and w leukemia strain (Ak/Rf) at 4 to 6 weeks of age, a single dose of 87r had no finite leukemogenic effect, though this dose was productive of ovarian neo- asms of the granulosa cell type, 175r was slightly and 350r markedly ukemogenic (33d)

There is no knowledge on the leukemogenic capacities of other radiating energy eir action is similar to that of x rays and they might also have a leukemogenic wer

*Coleukemogenesis* Leukemogenic agents to which man is exposed are numer- s and to understand the genesis of spontaneous leukemia it is of great interest find out if different agents which alone are not or are only slightly leukemogenic hance or retard the action of leukemogens X rays proved to be powerful -carcinogens (77, 72), and the following considerations led us to test their effect leukemogenesis After the application of x rays destruction of blood form- g organs is followed by regeneration during which mitotic figures are seen in undance It seemed probable that during this phase the blood forming organs

If the above assumption is correct, it may be expected that the Ak thymuses retain malignant potentialities after transplantation in a manner as limbs of creeper fowl embryos develop as achondroplastic when grafted on normal embryos It seems desirable to remove the thymus in strains of mice in which leukemia is myeloid or lymphoid but not thymic in origin and to study the effect of thymectomy on other neoplasms

Accidental involution of the thymus often accompanies malnutrition, intoxication or disease The experiments described show how these can modify an expression of genetic susceptibility to lymphoid leukemia

*Adrenal.* The reciprocal relationship between thymus and adrenal cortex is well known (cf 89, 22a, 22b) It follows that hyperplasia, hypoplasia or dysfunction of the adrenal cortex may modify the incidence of spontaneous leukemia or the response to a leukemogen However, there are no experimental studies on record on such an effect The conspicuous effect of adrenal cortical hormones on the growth of leukemic cells is discussed below

*Nutritional influence* Tannenbaum (108a, 108b) has presented statistical and experimental evidence indicating that restriction of caloric intake reduces the incidence of neoplasms, in man and animals Weight loss alone, whatever its cause, can be regarded as exerting an inhibiting influence on the incidence of neoplastic diseases

The effect of reduction in food intake on the genesis of spontaneous leukemia is indicated by the experiments of Saxton et al. (98) In mice of a high leukemia strain receiving limited amounts of a qualitatively adequate diet, the incidence of leukemia was 10 per cent as compared to 65 per cent in normally fed controls Bioassays at 9 to 12 months indicated the absence of malignant lymphocytes in the underfed mice, and disclosed their presence at 14, 16 and 17 months In normally fed mice of the same strain, malignant cells appeared at 7 to 13 months These experiments indicate that *a*, malignant lymphoid cells are absent in young normal mice of the high leukemia strain Ak, *b*, the malignant transformation takes place shortly before leukemia becomes manifest, and *c*, this transformation is delayed but not entirely prevented by underfeeding (98)

It is possible that the effects here described are due to some specific nutritional deficiency due to the overall reduction of food intake although none was noted Underdevelopment of thymus and lymphoid tissue might possibly explain a lowering of the incidence of leukemias but they can hardly account for the inhibition of other neoplasms by underfeeding Thymectomy produces a marked reduction in the incidence of leukemia without causing weight loss of the experimental animals (33c)

In mice "painted" with methylcholanthrene, a 50 per cent caloric restriction reduced the incidence of leukemia from 96 2 per cent to 35 per cent and increased the mean latent period from 73 to 237 days (115)

Lack of cystine in the diet also reduced the incidence of leukemia in mice 'painted' with methylcholanthrene The addition of gelatine to a low cystine diet raised the incidence of induced leukemia from 10 to 32 per cent and this effect is explained by the presence of a small amount of cystine in gelatine (116)

There are no data on the effect of low cystine diet on the incidence of spontaneous leukemia

Nutritional deficiency has been known to cause involution of the thymus Deficiency of riboflavin, pantothenic acid and thiamin or pyridoxin can cause depression of the weight of the thymus (106) Depression of lymphoid organs usually accompanies atrophy of the thymus and any agent which brings about atrophy of this organ might influence the incidence of lymphoid leukemia

*Chemical leukemogenesis* Earlier experiments suggested that tar and carcinogenic hydrocarbons are also leukemogenic (10, 25) Morton and Mider described a readily reproducible method to induce leukemia with such an agent They "painted" repeatedly a solution of methylcholanthrene on the skin of mice at different sites so as to minimize the development of local tumors This procedure, they believed, hastened the onset of leukemia in genetically susceptible strains of mice However, some low leukemia strains proved equally susceptible to this hydrocarbon

The quantity of methylcholanthrene sufficient to produce leukemia is small Six "paintings" with a 0 5 per cent solution of methylcholanthrene in benzol applied twice weekly over an area of a few centimeters has raised the leukemia incidence and hastened its onset Two paintings had little effect, eight paintings were better than six but more intense treatment did not raise the leukemia incidence (33d)

The efficiency of a hydrocarbon in hastening the appearance of leukemia in the high leukemia strain F bears a direct relation to the potency of the hydrocarbon to induce breast tumors (62d)

There are differences in susceptibility to leukemogens among different strains of mice (61) A strain resistant to the leukemogenic action of x-rays may be susceptible to that of methylcholanthrene and vice versa (60b)

The leukemias produced by hydrocarbons are usually of the lymphoid type and "atypical" (see below) There is no clear demonstration of the ability of these substances to induce myeloid and monocytic leukemias although there are experiments on record suggesting such potentialities (33b)

In most of these experiments benzol has been used as solvent Benzol itself is leukemogenic (cf 60b) but not under the conditions tested Solvents may have a pro or anti-carcinogenic action, though alone they do not produce new growth (12b)

The leukemogenic action of benzol is suggested by human observations (32), the few experimental studies on record suggest that it is a weak leukemogen (cf 25, 60b) The work of Büngeler et al (60b) suggesting that indol is leukemogenic remained unconfirmed

2-amino and 2 acetoaminofluorene when fed to rats produces neoplasms in different organs (118) including leukemias Bielschowsky (a) noted four leukemias in 104 rats of a non-leukemic strain fed on this compound but most of these rats had other neoplasms affecting the liver, mammary gland, etc , and these may have masked the leukemogenic potentialities of this compound

Later studies of Bielschowsky (b) suggest that as a coleukemogen this compound might be unusually effective

*Physical leukemogens* X rays have long been known to cause leukemia in man (cf 32, 52) and they produce it readily in mice According to a recent survey the incidence of leukemia in radiologists for a fifteen year period is ten times greater than in physicians who are not radiologists (70)

There is little known about the factors of irradiation responsible for the development of leukemia The doses which have this effect are large enough to destroy the bone marrow Regeneration follows atrophy but the stigmata of massive irradiation persist in experimental animals Hemopoietic tissues once massively irradiated may never be normal The spleen shrinks, connective tissue increases in amount and the organ is laden with hemosiderin filled macrophages Large areas of atrophy or necrosis have been a common autopsy finding in the femoral marrow of old mice that had been given about 350r at approximately six weeks of age (33d) Some cells among those newly formed acquire the property of unrestrained growth Both myeloid and lymphoid leukemias are produced by x rays Henshaw (b) has been most successful in inducing leukemia with x rays He has given doses of 200r for 3 to 4 month old mice, repeated at four week intervals for twenty weeks (total of 1200r) and thereby produced leukemia in 30 per cent of C57 mice Blood counts showed in general, leukopenia, but in some animals, recovery was followed by marked leukocytosis with leukemic infiltration at death Leukemia was predominantly of the primitive cell type and death occurred usually between the seventh and twelfth months However, the incidence of leukemia among his controls was also high (7 per cent) There is no study on record which would give quantitative information on the relation of the dose and quality of x rays and mode of application, to the incidence of leukemia Neither are the meager data on strain differences in susceptibility to x rays convincing It appears from the survey of the literature that x rays are weak leukemogens and act after a long incubation period In a small series, irradiation of F1 hybrids between a high and low leukemia strain (Ak/Rf) at 4 to 6 weeks of age, a single dose of 87r had no definite leukemogenic effect, though this dose was productive of ovarian neoplasms of the granulosa cell type, 175r was slightly and 350r markedly leukemogenic (33d)

There is no knowledge on the leukemogenic capacities of other radiating energy Their action is similar to that of x rays and they might also have a leukemogenic power

*Coleukemogenesis* Leukemogenic agents to which man is exposed are numerous and to understand the genesis of spontaneous leukemia it is of great interest to find out if different agents which alone are not or are only slightly leukemogenic enhance or retard the action of leukemogens X-rays proved to be powerful co-carcinogens (77, 72), and the following considerations led us to test their effect on leukemogenesis After the application of x rays destruction of blood forming organs is followed by regeneration during which mitotic figures are seen in abundance. It seemed probable that during this phase the blood forming organs

would be particularly susceptible to another leukemogen The experiments performed indicate that pre-irradiation with 175r enhances the susceptibility of mice to the leukemogenic action of small doses of methylcholanthrene, the leukemias appearing earlier and in larger numbers than in mice subjected to these agents alone (35a) Current experiments have shown that 87r applied under similar conditions are less and 350r are more effective than 175r in enhancing susceptibility to methylcholanthrene

The combined effect of x-rays and methylcholanthrene was tested by Kaplan and Kirschbaum under entirely different conditions X-rays were given in doses of 80r until a total of 720r and 880r were given The application of methylcholanthrene was so intense that 80 per cent of the mice died of leukemia at an average of 114 days as contrasted with 58 per cent of mice exposed to both agents, dying at an average of 127 days The authors are inclined to explain the apparent discrepancy between their results and ours (35a) by differences in the genetic constitution of the strain of mice used, but they also emphasize the higher dosage and different method of irradiation in their experiment As Tannenbaum points out, cocarcinogenic and anticarcinogenic actions are usually effective only during one stage of carcinogenesis (108c) The procedures of the two groups of investigators are not comparable and these may account entirely for the differences in the results

There are other leukemogens whose co-operative potencies deserve further study, e g , estrogens are known to be leukemogenic and carcinogenic in large quantities, and procarcinogenic (43) in small quantities Among the leukemogenic substances the pluripotent acetoaminofluorene has a remarkable carcinogenic action on stimulated cells It readily produces carcinoma of the thyroid gland that had been rendered hyperplastic with thiouracil (7b) Any agent that stimulates hemopoietic organs may prepare the ground for a leukemogen, and may cause neoplasia of blood cells when small doses of a leukemogen also reach hemopoietic foci Hereditary susceptibility to leukemia may be localized to one lymphoid organ as suggested by thymectomy experiments (84) However if an organism is exposed to large quantities of a carcinogenic agent, neoplastic cells probably arise in many foci, e g , methylcholanthrene produced innumerable discrete lung tumors in almost every mouse that had been painted with leukemogenic doses of this hydrocarbon

*Urinary substances stimulating leukopoiesis* The idea that leukemia may result from disturbance of the unknown mechanism which maintains leukocyte level will now be considered Stimuli producing transient depressions and elevations of this level are well known and it is conceivable that a disturbance of this mechanism similar to that of an endocrine gland might result in a lasting rise of the leukocyte level and in an accompanying hyperplasia of a hemopoietic system Thus, a leukemia-like change might result but this supposition does not explain the change in the immature blood cells endowing them with unrestrained growth in new hosts into which they are grafted

Wearn et al (cf 53, 50) discovered that the urine of leukemic patients contains substances that stimulate hemopoiesis in guinea pigs Miller and Turner

(75, 110) believe that these substances are specific stimulators of hemopoietic systems All of 36 animals given this principle from patients with chronic myeloid leukemia are stated to have developed changes similar to that of human myeloid leukemia Their figures 1 to 3 illustrating these changes show, in the reviewer's opinion, hemopoiesis not definitely leukemic in character All of 40 animals given the principle from patients with chronic lymphoid leukemia are described as having developed comparable changes In 6 guinea pigs given the material from patients with Hodgkin's disease, a "trend toward lymphoid stimulation was apparent with the character of lymphosarcoma in some animals" The material with lymphoid activity is believed to be a hydroxy-acid, that with myeloid activity a non-carbinol acid Patients with monocytic leukemia and Hodgkin's disease are excreting both in large quantities but one substance can be converted into the other Normal people are assumed to excrete similar substances in small quantities although none has been demonstrated The most potent substance that produces myeloid hyperplasia and metaplasia in guinea pigs is in the urine of patients with myeloid leukemia The active principle can be concentrated by the benzoic acid absorption technic A different substance with similar activity obtained by Hirschman et al seems to be a protein or glycoprotein

No attempt is described by these investigators to prove transmissibility of the disease by cells Leukemia of the guinea pig is known to be transmissible (25) as are most, if not all, leukemias of mice The effect of similar extracts from the urine of patients with other neoplasms has not been adequately tested In mice, advanced non malignant extramedullary hemopoiesis frequently accompanies tumors of different sorts (65, 4) The animals of Miller and Turner usually became moribund at 3 to 5 weeks after the beginning of the treatment—an unlikely brevity and course for a neoplasm.

These authors interpret their data as supporting the well known "theory" of Ziegler, which postulates a balance between myeloid and lymphoid tissues and hyperplasia of one system consequent to hypoplasia of the other but until better evidence is presented this "theory" will remain an assumption Wearn and his present associates regard the changes described as leukemoid but such changes are non-specific The chemical and biological properties of substances producing them and their relation to lymphoid and myeloid leukemias deserve further study

*Leukemoid reactions* The hemopoietic reserve of the bone marrow appears less in rodents than in man, and it is common to note orderly myelo- and erythropoiesis in the liver and spleen of older rodents and during the course of varied infections and intoxications Most of the greatly enlarged spleen and less often as much as one-third of the enlarged liver may be hemopoietic tissue and the leukocyte count may exceed 200,000 Indeed, in extent, these hemopoietic foci may equal that encountered in the usual cases of leukemia These hemopoietic cells are, however, not autonomous and they cannot be grafted on normal related mice They also lack the invasive character of malignant blood cells Non-malignant extramedullary hemopoiesis (4, 65, 90, 33d)

often accompanies chemical carcinogenesis and grafted tumors of different sorts, particularly when the latter are large and undergo necrosis or secondary inflammation With removal of the grafted tumor and healing of wounds, or cessation of causative infection, they regress The morphological criteria differentiating it from leukemias have been well described by Barnes and Sisman Occasionally it is not possible to distinguish between these two fundamentally different processes by microscopic examination Numerous bioassays were performed to exclude the leukemic character of this extramedullary hemopoiesis and with rare exception the inoculation into young mice of the homologous strain failed to yield leukemia (33d) It is possible that this advanced hyperplastic change is mildly preleukemic

*The leukemic cell* Almost all investigators of experimental leukemia arrived at the conclusion that the essential change in leukemia is a malignant transformation of an immature blood cell and that a single locus of lymphoid neoplasia can produce as an end result either leukemia or lymphosarcoma, depending on the character of the malignant lymphocytes The factors responsible for tissue invasiveness and degree of blood involvement are unknown (33b, 60b) It is possible to produce tumor-like masses in the subcutaneous tissue by the introduction of malignant blood cells in the subcutaneous tissue even from cases with diffuse involvement, formerly spoken of as systemic disease, not accompanied by tumor formation (33d)

The idea that the basic change in leukemia is one of interference with maturation of primitive cells with consequent proliferation lacks experimental support In Potter's opinion the malignant transformation is "the result of a depression in the rate of differentiation which accumulates cells" (cf 83) But in some myeloid leukemias there is a conspicuous maturation and this view does not explain the essential alteration in leukemia which takes place in the cell

The idea that lymphocytes with leukemic properties in one animal may behave as normal cells on transplantation into an animal with a different genetic constitution (80b) has no basis Experimental data indicate that leukemic cells under such conditions do not proliferate and the studies of Loeb imply that they are likely to be destroyed by the inflammatory reaction provoked by the genetic differential between host and recipient There are no data on record that suggest that leukemic cells re-acquire the basic properties of a normal cell under any condition If such a process should occur it would indicate a possible means of controlling neoplastic growths

The experimental observations indicate that the leukemic cell is a newly formed cell with individual properties of its own often perplex in terms of normal maturation, e g , in a malignant myeloid cell (as is common in myeloid leukemias of chickens) the nuclear lobation may be advanced, yet granulation is scanty if any One type of leukemic cell in mice is characterized by exaggerated nuclear lobations, absence of cytoplasmic granules and of oxydase enzymes and lack of phagocytic ability This was first described as an "atypical leukemia" (3a) but subsequent observations have shown this to be a fairly characteristic type although its genesis and character is subject to controversy (66) Occa-

sionally the typing of leukemias in man and mice offers considerable difficulty Organ affinities are of little help in their identification since any blood forming organ can be the favored site of any type of malignant blood cell In transmission experiments the three types of blood cells (lymphocytes, granulocytes and monocytes) remain true to type and transitions or transformations have not been observed These observations support the idea of independence of these cell types in the adult animal

Neoplasms arising in reticular or endothelial cells of hemopoietic organs have also been observed in rats (29) and in mice and transmitted in series (3b, 39) These cells remain true to type and fail to produce any type of blood cell One of these could be readily grafted on the chorioallantois of the chick embryo producing a bulky growth in eight days There seems to be a relation between neoplasm of monocytes or reticular cells and Hodgkin's disease (33a) A cytoplasmic inclusion body in lymph nodes characteristic of Hodgkin's disease described by Grand deserves further attention

It is beyond the scope of the present review to survey in detail chemical, metabolic and cytological studies describing differences between normal and malignant blood cells There is no test tube reaction which would indicate malignancy The differences noted between normal and malignant have been proven to be quantitative rather than qualitative and it is uncertain whether they are related to the cause of the malignant transformation or result from it Claude fractionated extracts of malignant rat lymphocytes by means of centrifugation and has shown that ribose nucleic acid occurs in the cytoplasm of the leukemic cells only in association with formed elements of relatively large size, namely, microsomes and mitochondria (15a) These exist as physical entities and their structure is well shown by the electron microscope (16)

*Tissue cultures* The abnormal characteristics of leukemic cells of mice are maintained in tissue cultures Each cell type has a characteristic motility and this may help to identify the cell type (67) In addition, each malignant line of the same cell type studied proved to possess individual characteristics perpetuated in tissue cultures The leukemic lymphoid cells studied (67) migrated from the explants more slowly than normal cells, showing characteristics of malignant cells in that they were larger and had larger and more granular nuclei than normal lymphocytes The malignant monocytes studied (67) behaved in tissue cultures as the epithelioid cells of the tubercle. They were phagocytic, ingested red blood cells and formed bilirubin Their nuclei were somewhat larger and had more chromatin granules than those of normal epithelioid cells (67)

Lewis and Lewis, and Bichel cultured malignant lymphocytes of mice over periods of months but no one has succeeded thus far in growing in vitro normal myeloblasts or lymphoid cells while leukemic cells seem to grow with ease.

Earlier investigators (cf 33b) described maturation of human leukemic myeloid cells in vitro in the manner of normal immature myeloid cells This observation, partially confirmed by Israëls is conflicting with those made on myeloid leukemia of mice and since it suggests a maturation defect as the basic

change in human leukemia, the discrepancy requires a clarification Israëls goes as far as denying the applicability of experimental studies in rodents to human leukemias citing opinions and observations in favor of the non-neoplastic nature of human leukemias, but none of these appears conclusive This investigator has cultured cells from the bone marrow from one case of myeloid leukemia and three cases of monocytic leukemias and noted maturation of the presumably leukemic cells Such studies are liable to the pitfalls of interpretations made on the basis of fixed and stained preparations, they should be documented with sections and motion picture records

The chorioallantoic membrane of the developing chick embryo provides a medium in which human leukemic leukocytes may grow for a short period (93a) Thiersch noted the development of hyperplastic osteosclerosis in fowls which hatched after inoculation with material from human leukemias None of the fowls or embryos had leukemia The development of these bony changes may not be a mere coincidence (cf 56)

*Metabolism of leukemic cells* Most earlier studies are subjects of a technical error The circulating blood is seldom if ever a pure culture of malignant blood cells, and their number is variable and uncertain Leukemia often produces a leukemoid change and malignant blood cells may be indistinguishable in appearance from their immature normal homologues In working with tissues the non-malignant framework cells and blood components and other residual normal or injured cells of the host have not been given proper consideration (11, 12b, 111)

The anaerobic glycolysis of lymph nodes and spleens with induced leukemias showed no definite metabolic distinctions between control and leukemic mice irrespective of the type and extent of infiltration The anaerobic glycolysis of livers, however, showed a two- to eight-fold increase depending upon the extent of infiltration All three types of analysis, metabolic, histologic and transmission concurred, indicating a relatively sudden onset and rapid progress of leukemia a few months after painting the mice with methylcholanthrene (11)

The respiratory quotients of all tissues examined were below unity The aerobic glycolysis values of preleukemic, leukemic and leukemoid lymph nodes, spleens and livers were 50 per cent to 100 per cent above normal No evidence was obtained for the existence of a preleukemic metabolism different from normal In the opinion of Burk the metabolism of induced mouse leukemia seems to represent the lowest limit of a type of metabolism of a malignant tissue (11)

The presence of a neoplasm influences a wide variety of biochemical processes in host tissue remote from the site of the tumor (12a, 12b, 111) The leukemic state may alter the metabolism of liver cells (48) Hall attributed the increase in anaerobic glycolysis rate of livers of leukemic mice to two factors *a*, the presence in the liver of malignant lymphocytes that are carrying on anaerobic glycolysis at a rate similar to that of the same lymphoid cells in the lymph nodes, *b*, to a considerable increase in the glycolytic activity of the parenchymal cells of the liver The latter appears to be due to the leukemic state and may be independent of the presence of leukemic cells in the organ

Normal bone marrow cells other than erythrocytes fulfill all but one criterion of a tumor type of metabolism of Burk, the relatively high R Q (about 0 96) distinguishing them from malignant cells (112) The available data on R.Q values are insufficient for generalization and the studies of Warren (112) indicate that no known metabolic reactions serve to distinguish malignant from non-malignant hemopoiesis

Normal cells appear to utilize less pyruvate and usually convert a greater proportion of pyruvate to lactate than leukemic cells (1)

*Histogenesis of the leukemic cell* The ease of induction of leukemia and the availability of strains in which the disease is almost inevitable at a given age, enable a study of its histogenesis Unfortunately, observations on the interrelation of cell types on the basis of appearances in fixed and stained preparations proved undependable and so are these histological studies on the origin of leukemia.

The tissue culture studies and transmission experiments indicate that the leukemic cells are capable of proliferating indefinitely, and it is conceivable that the malignant change affects the homologous cell the lymphocyte, myeloblast or monocyte However the reticular framework cells of the blood forming organs are widely regarded as the source of both normal and leukemic cells Potter et al (94) described a localized reticulum cell hyperplasia in the high leukemia strain C58, preceding leukemia This is particularly conspicuous in the medullary regions of the lymph nodes and perivascular regions of the liver, and is absent in low leukemia strains examined at comparable ages. They concluded that the first malignant lymphocytes are produced by reticulum cells Since this reticulum cell proliferation is widespread it is assumed that not all become sources of leukemic cells In the low leukemia strains studied by the reviewer, reticulum hyperplasia is common with advancing age (35b) and is frequently associated with non malignant extramedullary hemopoiesis There seems to be an association between hemopoiesis and reticulum hyperplasia but it is possible that the same stimulus provokes both independently, or that the reticulum cell proliferation is secondary One of the most common sites of reticulum cell hyperplasia is in the liver, yet this organ is an extremely rare site of lymphosarcoma and may show only scant involvement in the early stages of leukemia In the Ak stock the thymectomy experiments suggest that the thymus is the common site of the primary leukemic change The origin of malignant blood cells in reticulum requires better evidence than hitherto presented

The best index for the leukemic character of hemopoiesis in a given organ is a positive bioassay Bioassays made during the preleukemic phase of spontaneous or induced leukemia have disclosed the presence of malignant blood cells not suspected on histological examination The solution of this problem may come from further systematic studies with non-leukemic and leukemic strains correlating histological changes with bioassays

THE GROWTH OF LEUKEMIC CELLS Neoplastic cells are usually designated as autonomous or unrestrained but these are only relative terms Physiological

and other stimuli influencing normal hemopoiesis influence to a limited extent the growth of leukemic cells and numerous recent investigations are concerned with factors influencing their growth. Unfortunately, many of these investigations are of little value because of the lack of consideration of the growth interference occurring when there is a genetic differential between the leukemic cells and the host on which they are grafted

*Genetic and antigenic considerations* The growth of a leukemic cell in a host other than that in which it has arisen is subject to a restraint, varying in intensity with the magnitude of the differential between grafted cells and the hosts The following are illustrative examples of the difference between the genetics of spontaneous and transmitted leukemia In C3H mice, leukemia is almost non-existent, yet this strain is highly susceptible to the growth of cells arising in the high leukemia strain Ak (36) F1 hybrids between high and low leukemia strains have a moderate or low incidence of spontaneous leukemia, yet they are with rare exceptions as susceptible to leukemias of the high leukemia strain as members of the latter (36) Accordingly, experiments dealing with the genetics of transmissible leukemia have little, if any bearing upon problems of inherited susceptibility to this disease Experiments on the induction of resistance to transplantable leukemia and modification of such resistance by chemical, physical or other agents are concerned with factors of transplantation and have little if any bearing on the course of spontaneous leukemia, e g , a transmissible rat leukemia can be grafted on only about 80 per cent of rats of the stock of origin indicating the existence of a differential between host and leukemic cells (79b) Embryonic tissue produces resistance to grafts in about 85 per cent of the rats treated Several chemical agents as well as x-rays are capable of breaking down this resistance (79b) Such experiments are not concerned with the growth of spontaneous neoplasms

Leo Loeb, in a classical monograph on individuality has described the reaction of the body to alien cells introduced The individual differential acts as inflammatory irritant and provokes the formation of antibodies in the new host Tumors grafted on unrelated mice seldom take, and if they take they often regress Tumors which take in unrelated mice often tend to regress after application of an agent somewhat damaging to the tumor cells Such experiments raise the questions Have the agents used altered the differential between host and recipient cells, depressing the reactivity of the host or increasing the antigenicity of the graft, or have they acted as a growth stimulant or depressor and could they act also in the absence of antigenic differentials against a spontaneous malignant tumor Transplantation of leukemias with a differential between host and grafted cells contributes to an understanding of the individuality of the leukemic cells but does not directly disclose factors influencing the growth of spontaneous neoplasms In some cases, immunization processes are apparent only after the growing neoplasm has been completely extirpated (Loeb, p 405) Agents injuring the tumor may augment immune reactions resulting in a "cure" or decrease of the per cent of takes

Accordingly, the most conclusive experiments on growth modifiers are those

conducted on spontaneous or induced neoplasms Unfortunately, the leukemias of animals are not recognized early enough The nearest to an ego is an identical twin, and the nearest to an identical twin are littermates of inbred mice Continued inbreeding leads to homozygosity though the latter may never be attained The use of such strains proved trustworthy in orientation studies for growth modifiers

Unfortunately, leukemic cells may change in the course of successive transplantation The cells often gain in proliferative vigor and in the course of years or months all chronic transmissible strains of the reviewer have ended up as acute, producing a fatal disease in 5 to 12 days instead of months At the same time there was but slight or no change in the appearance of the leukemic cells

In the course of transplantation by MacDowell et al in inbred strains of mice, antigenic differences became evident between grafted cells and hosts with production of immunity against these cells This immunity did not interfere with the genesis and growth of leukemic cells in "immunized" mice (81a)

MacDowell et al have noted that by introducing small numbers of leukemic cells arising in the homologous strain the mouse may survive and tolerate increasingly larger numbers of cells Hosts susceptible to tumor grafts can be protected also passively by treatment with minced tissue containing living cells from an actively immunized animal Normal and embryonal tissue from an unrelated mouse may also confer immunity Tissue of the same genetic constitution as the host is ineffective. Circulating antibodies play an important part in immunity to transplants of leukemic cells (44)

Leo Loeb believes that there is no individuality differential between normal and cancer cells of the same animal and immunotherapy of neoplasms is, therefore, not feasible If there is a subtle genetic difference between a spontaneous neoplastic cell and the homologous normal cell of its host, as is suggested by proponents of the somatic mutation theory, it is not of an order to be antigenic in the host, otherwise it would be destroyed It is possible that limitations of spread in spontaneous leukemias and failure to circulate in the blood stream in large numbers are due to slight antigenicity, but even this has thus far not been proved The numerous immunity studies to prepare, by specific absorption, a solution acting specifically on malignant cells have been carried out with grafted tumors, and are, therefore, not applicable to spontaneous disease

Blood cells would seem to be particularly suitable for the study of specific antibodies for malignant tissue proteins but such studies have thus far not been reported. Opinions on the subject are varied MacDowell et al consider immunization against spontaneous leukemic cells a possibility while for reasons outlined, the reviewer regards this as most unlikely

In evaluating the experiments on modification of the growth of leukemic cells most informative are those conducted with spontaneous leukemia Large numbers of spontaneous cases are necessary for the study of each factor because different lines of leukemic cells of the same type may vary widely in reactivity

to the same agent (31) This is well known for human neoplasms, e g , the hormonal response of prostatic carcinoma seems to be lasting in less than 25 per cent of the cases, and is transient in 75 per cent Next in value are studies made with transmissible leukemias arising and transplanted in a highly inbred strain Leukemic cells vary greatly among themselves, hence, the use of several different lines is desirable A given line of transmissible leukemia reacts uniformly to the same agent For preliminary experiments, transmitted leukemias in well inbred lines may be more suitable than spontaneous leukemias The onset and course of the leukemia is known with transmitted leukemias, while spontaneous leukemias are only available at the advanced stage and their course is unpredictable The greater the differential between grafted cells and hosts, the less informative is the experiment Tendency for regression or failure to take in 100 per cent of the animals (discounting technical errors) are indications of individuality differentials

*Hormonal growth factors* The female sex hormones and adrenal cortical hormones undoubtedly affect lymphopoiesis There is ample evidence indicating that the latter inhibit the growth of some malignant lymphocytes while the former have not been tried on a comparable scale

*Adrenal cortical hormones* Heilman and Kendall have shown that 11-dehydro-17 hydroxycorticosterone (compound E) produces atrophy of thymus and of lymphoid tissues When given to mice with a transmissible lymphosarcoma, it caused rapid regression of the growth, but in most animals regression was incomplete and temporary, and after an interval of a few days or weeks the growth recurred Gradually the leukemic cells became refractory to this hormone and eventually the tumor caused the death of every mouse Resistance to the hormone resided in the tumor cell for the resistant tumor transplanted to another animal remained resistant A few mice which were given the cells of this tumor by intravenous inoculation were also treated with compound E but prolongation of life of the mice with systemic leukemia was slight A reticulum cell sarcoma did not respond at all to this compound Several transplantable mammary carcinomas were tested and only slight retardation in growth was observed

In experiments on the effect of adrenalectomy on the susceptibility to transmitted leukemia, Murphy and Sturm used middle aged rats of a substrain of Wistar rats that are only partially susceptible to leukemia arising in this strain Only 46 per cent of 32 inoculated animals developed the disease Adrenalectomy has raised this figure to 90 3 per cent (79a) This effect may be entirely nonspecific and may mean no more than lowering the resistance of the host to grafts Adrenalectomy produces stimulation of lymphoid activity and the latter should raise resistance to tumors, according to the theory of Murphy (78a) Sturm and Murphy (107) attribute the greater receptivity of adrenalectomized rats to transplanted leukemia to the same factor which stimulates lymphoid tissue It is possible, however, that the effects of adrenalectomy are non-specific in that the growth of other neoplasms would also be enhanced by adrenalectomy In other words, this drastic procedure might break down the

32685

differentials existing between host and grafted cells The resistance of adrenal ectomized rats is known to be markedly depressed to toxins, poisons, bacterial and protozoan infections (92)

The inhibitory effect of adrenal cortical and pituitary adrenotropic hormones on the growth of transplanted leukemic cells was tested by Murphy and Sturm under the condition mentioned From 0 to 10 per cent of inoculated controls failed to come down with leukemia as contrasted with about 20 to 60 per cent of those receiving the hormone (79c)

*Female sex hormone* Experiments on the relation of sex hormones to the induction of leukemia already reviewed do not suggest that these hormones, given in physiological amounts have a marked effect on the proliferation of the malignant cells There are no reports on the effect of subfatal quantities of these hormones on the growth of leukemic cells Stilbestrol, given in large quantities, brings about atrophy of thymus followed by that of other lymphoid tissues and it retards the growth of mice (41) Estrogenic hormones are one of the few substances which will affect the lymphoid tissue in the absence of adrenals (101)

Little is known about the effect of other hormones on malignant blood cells Induced myxedema did not alter the course of chronic lymphoid or myeloid leukemia in man (91)

*Nutritional factors* Quantitative restriction of a qualitatively adequate diet resulted in a slight prolongation of life with transmitted lymphoid and myeloid leukemias, but death from leukemia was not prevented (31) Under feeding was begun early in the course of the disease and was about as drastic as the mice could stand Nevertheless, prolongation of life was less than that resulting from application of x rays, benzol or arsenicals (31)

*Nursing influence* The milk of certain lactating female mice promotes the growth of some transplantable lymphoid and myeloid leukemias in normally refractory mice (63) This influence was present throughout the period of lactation and was maintained through three generations in normally refractory mice The factor is present in saline extracts from liver, spleen or mammary gland, dialyses through parchment membrane resists 85°C for 20 minutes and 50 per cent glycerol for 30 days (63) The existence of such a factor analogous to the agent of mammary carcinoma is a challenge to the genetic theories of transplantation and throws new light on growth factors of neoplastic cells

*Chemical growth modifiers* (Experimental chemotherapy of leukemia) Following the ideas of Peacock and Beck and of Haddow that a carcinogen may also inhibit the growth of neoplastic cells, Stamer and Engelbreth Holm (105) tested the effect of intravenous injection of a colloidal suspension of 9,10 dimethyl 1,2 benzanthracene on transmissible leukemia Small doses of this substance prolonged the life of the mice with transmitted leukemia and a large dose actually controlled it in all of five mice that had been given this treatment It is noteworthy that this dose is fatal by the subcutaneous or intraperitoneal routes and that no tumors appeared after intravenous injection of this carcinogen These experiments require confirmation and extension Are other transmitted

and spontaneous leukemias affected by 9,10-dimethyl-1,2 benzanthracene, do other more potent leukemogens have a similar effect, are compounds related chemically but lacking carcinogenic potencies also effective in controlling the growth of leukemia, what is the action of this compound on normal hemopoiesis—are some of the questions to be answered by experiments

Flory et al (31) have tested numerous organic compounds using different strains of transmitted leukemia and found that different leukemias respond differently to the same agent but the effect of a given chemical on a given strain is fairly constant Benzol doubled or tripled the life span of mice with transmissible chloroleukemia and occasionally arrested the disease Its effects were manifest even when administered during the advanced stage of the disease Other types of leukemias tested responded little if at all to benzol and no benzol derivative was found that would be more effective than benzol (31) Knowledge of the fate of benzol in the body and its mode of action might aid in the extension of these findings Potassium arsenite prolonged somewhat the life of mice with certain transmissible leukemias No organic arsenical tested had a stronger effect and none prevented death from leukemia (31)

Chambers et al studied the effect of several compounds which inhibit the respiration of malignant lymphoid tissues in vitro, on the growth of normal and malignant lymphoid cells in tissue culture These include N,N,N$^1$,N$^1$-Tetramethyl-o-Phenylenediamine which was about seven times more toxic to malignant than to normal lymphoid cells In general, the malignant cells were of the more immature type and it is possible that immaturity per se was the cause of excessive sensitivity (14) In tissue cultures lymphoid sarcoma cells of rats were more susceptible to heptanal sodium bisulfite methylsalicylate than normal lymphoid cells (Cameron et al ) It remains to be shown whether these short-term tissue culture studies yield results that parallel those in vivo Heptylaldehyde bisulfate strongly depresses respiration of lymphocytes in vitro but it had no effect on lymphomatous tumors of rats and on leukemic patients (102) Colchicine temporarily inhibited the growth of transmitted lymphosarcoma (68, 117)

*Physical agents* Lymphomatous tissue retains more radioactive phosphorus than normal lymphoid tissue from 1 to 19 days after administration of tagged phosphorus The exchange (specific activity) of phosphorus in the lymphoma studied was twice as great as in normal lymphoid tissue (100) In rapidly growing lymphomatous tissue the nucleoprotein fraction was found to contain 90 to 95 per cent of the total nuclear radioactive phosphorus From the rate of its uptake it was calculated that a new lymphoma nucleus was synthesized once every 27 hours The great accumulation of P32 in malignant cells is attributed to an increase in mitotic activity (71a) Warren found that administrated radioactive phosphorus is retained in greatest amounts in those organs of leukemic patients which are heavily infiltrated with leukemic cells Slowly metabolizing tissues contain but little phosphorus The distribution of radioactive phosphorus in man is of approximately the same order as in rodents (113a) Radioactive phosphorus is a useful means of therapy of leukemia

because of its preferential concentration in rapidly proliferating cells, hence in leukemic infiltrations (113b) If given many days before death, the concentration of P32 is greatest in osseous tissues while if given just before death, most is found in the bone marrow, lymph nodes, spleen and liver This suggests that it is first utilized by more rapidly metabolizing tissues and by those most frequently infiltrated with leukemic cells and later by bones (28) Numerous other studies with different radioactive elements, neutrons and x rays have been reviewed by Lawrence

*X rays* Of all agents tested by Flory et al , x rays had the broadest range in destroying malignant blood cells They prolonged the life of most leukemic animals but in no case has it been possible to destroy all malignant cells without doing fatal damage to normal organs

Following exposure to x rays and to neutrons there is a sharp increase in abnormal mitoses 3 hours after exposure and a greater increase at 12 hours. As the dose of the x rays increases the percentage of mitoses decreases indicating (Marschak, 71b) that the damage results from a single encounter of the x rays with a sensitive part of the chromosomes Unlike those of other cells studied, chromosomes of a mouse lymphoma are sensitive to x rays and neutrons not only at the onset of the prophase but also during the period of the resting stage where the sensitivity is even greater (71b) Since neutrons are more efficient than x rays in producing chromosome abnormalities in the nuclear resting stage, Marshak predicts that they will produce regression of neoplasms resistent to x rays (71b)

*Cold* Exposure of starved leukemic mice to cold at 6–8°C , during 5 to more than 24 hours, caused degeneration and disappearance of leukemic lymphocytes but the leukemic infiltrations reappeared in all animals that survived this drastic treatment (26) It is noteworthy in this connection that malignant lymphocytes resist freezing (9)

AVIAN LEUKOSIS AND THE VIRUS PROBLEM. The virus theory of leukemia is based mainly on experiments with avian leukemia (leukosis) and the latter will, therefore, be briefly reviewed The problems of avian leukosis are identical with those of avian sarcoma Numerous viruses have been isolated which produce either leukosis or sarcoma or both The range of each virus is constant, however, under usual conditions Earlier experiments suggesting a change in their affinities have not been confirmed Duran Reynals has shown that the agent of chicken sarcoma is capable of profound modification when passed through another species but it is uncertain how far leukemogenic properties can be acquired or lost in this manner

Three main types of avian leukosis are known, erythro- and myeloleukosis, which are usually caused by the same agent, and lymphoid leukosis, which can be transmitted with difficulty or not at all. The latter occurs in many forms and is probably caused by several different viruses. An avian lymphoma readily transmissible by cellular grafts, has been described by Olson No cell free agent has been demonstrated in this lymphoma but no intense search seems to have been made for such an agent. Numerous investigators have induced sarcoma in chicks with hydrocarbons but it is debatable if these tumors

contain an agent (virus) Nobody has described the production of leukosis in chickens by pure carcinogenic chemicals Immunological studies suggest the presence of a virus in a chemically induced chicken tumor that is not transmissible by cell free material, sera of fowls bearing such tumors neutralize a leukosis-sarcoma agent (Gottschalk) The direct demonstration of an agent in chicken leukosis or sarcoma is sometimes difficult and the studies of Rous et al and of others indicate that no avian tumor or leukosis can be certified to be free from such agents A non-neoplastic change, osteosclerosis, is believed to be caused by some agents of leukosis (56)

A characteristic form of avian leukosis associated with infiltrations in the nervous system commonly called fowl paralysis or neurolymphomatosis is of great economic importance and has been studied extensively in numerous places It appears to be sometimes neoplastic and sometimes inflammatory in character with predominance of small lymphocytes Numerous transmission experiments yielded suggestive evidence of its transmissibility until recently Blakemore discovered that it is caused by a filter-passing agent which produces a non-fatal myocarditis in baby chicks Neurolymphomatosis is conceived to be a late phase of the same disease There are distinct hereditary differences in susceptibility to agents of fowl leukosis and sarcoma (54, 114) Leukemic cells placed on the chorioallantois produce leukosis in chick embryos but the cell free virus does not although it persists in the embryo (93b) In tissue cultures the agent of fowl leukosis could be grown in the presence of fibroblasts (21) Thus this agent is capable of multiplication in the presence of cells which it does not render neoplastic

For years, the discussion centered around the question whether the cause of avian leukosis was a virus similar to that of other contagious diseases or an autocatalytic endogenous selfperpetuating substance inducing other cells to an irreversible neoplastic change Neither the morphological characteristics nor behavior in the body would distinguish a virus-induced leukosis or other neoplasm from one seemingly free from virus There are strains of avian leukosis and sarcoma readily transmissible by cell free agents, strains transmissible by cells only and all gradations in between This virus can be conceived as part of the reproductive matter of the abnormal cell (20, 19) and the fact that it is separable from the cell makes it a useful tool for the study of cell determinants Normal cells also contain in their cytoplasm sedimentable substances of the order of magnitude of viruses Chemical and immunological studies suggest a similarity between virus and normal heavy tissue particles (15b) They are carriers of numerous enzymatic activities (cf Kabat) It is possible that the virus is a modified normal heavy cytoplasmic substance (Claude) or that the so-called virus thus far analysed is essentially normal heavy material and only a small fraction of it is virus (Kabat) The trend is to consider these viruses as essential but altered components of the heavy material present in normal cells and to regard them as autosynthetic as are genes and some enzymes The carcinoma protein is a modified normal protein with changed enzymatic activity occasionally containing a "reproductive particle" (V R Potter)

The experiments of Duran-Reynals on modification of an avian neoplastic

virus through passage of different species are explained by assuming that the new environment provides different "building blocks," causing a reconstruction of the virus, the altered virus becoming dominant in the new host (Dixon) Needham compares carcinogenesis by virus with neural plate induction The latter could also be propagated by cell free extracts The synthesis of further supplies of evocators may be stimulated autocatalytically like trypsin from trypsinogen or plant viruses in their hosts

In the light of these investigations, neoplasia in mammals can be explained by a similar cytoplasmic disturbance yielding a virus-like agent Engelbreth Holm and Frederikson described experiments suggesting such an agent in mouse leukemia, but their findings still await confirmation This agent, they thought, is rapidly inactivated by oxidation

THE SOMATIC MUTATION THEORY The idea that the neoplastic change is analogous to a somatic mutation has had its proponents and opponents for several decades (36) All somatic cells of an organism are believed to have an identical chromosomal make-up with a complete set of genes responsible for the organismal differentials Differentiation or transformation of one cell type into another is not accompanied by chromosomal changes When the somatic mutation theory was proposed, only chromosomes were regarded to be hereditary components of the cell Subsequently, cytoplasmic materials have been recognized that are self reproducing and important in determining the characteristics of the cell The term somatic mutation will be used here in a broad sense, meaning changes in any component of the cells which is self reproducing and determines cellular characteristics An acceptance of the idea that the neoplastic change is analogous to a somatic mutation requires proof that the change resides in the chromosomes or in reproductive cytoplasmic particles No such changes have thus far been noted in the chromosomes of leukemic cells, and nothing is known about the hypothetical reproductive particles in the cytoplasm of these cells

Indirect evidence for a genetic basis of the leukemic alteration is suggested by transplantation tests Transplantation tests in highly inbred strains are believed to yield information on the genetic character of the cells Hybrids of known genetic composition have a greater assortment of chromosomes than homozygous inbred mice and transplantation tests in F1 hybrids yielded data of greater value It has been known that grafts of normal cells of F1 hybrids succeed 100 per cent in F1 hybrids but not at all in parental mice This was verified for spleens of the F1 hybrids of a high (Ak) and a low (Rf) leukemia strain in which these transplantation experiments were made (58) Numerous lines of leukemic cells arising spontaneously in F1 and other hybrids of these strains were grafted on parental and on hybrid mice Unlike normal spleens they proved readily transplantable to mice of the parental high leukemia mice (99) Most leukemias induced by methylcholanthrene in F1 hybrids of these strains could not be grafted on parental mice but some could be grafted on either or on both parental strains (34)

These results are explained by Loeb by assuming that F1 cells contain, in addition to the gene sets derived from the parents which determine the "or

ganismal differentials" of the leukemic cells, an intrinsic stimulus (named by him Gi—intrinsic growth factor) which is derived from the parent susceptible to the development of spontaneous leukemia This intrinsic stimulus is responsible for the conversion of normal lymphoid cells into leukemic cells In the high leukemia strain the existence of an auxiliary extrinsic growth promoting factor (Ge) is postulated, which makes it possible for leukemic cells to multiply This hypothetical Ge factor is not identical with the nursing influence of Law (already described) since the former is transmitted by both mother and father It may represent gene hormones through which in the course of embryonal development, gene effects may be transmitted to the recipient tissues (Loeb, p 383)

Not all leukemic cells arising in F1 hybrids behaved in the manner described Kirschbaum and Strong (62b) noted that leukemic cells arising in CBA x F hybrids behaved in transplantation tests as normal spleens

Mutations are believed to be sudden while the neoplastic change is stated to be a step by step non-genetical process This, however, may be more apparent than real, as was recently pointed out by Mottram for cancer cells, the more rapidly proliferating cell of a heterogeneous malignant cell population gradually outnumbering the less rapidly growing cell lines Another objection to the somatic mutation hypothesis is that malignant cells frequently change in the course of transplantation Malignant cells, as other living cells, are subject to inheritable modifications but, since the use of inbred strains of mice, the only consistent change noted in lines of malignant cells is that in growth vigor but not a change in character

When a normal cell turns malignant it can survive and proliferate only when the deviation in its components is not of sufficient magnitude to create a new antigen The formation of a new substance is not necessary for antigenicity, structural differences create antigenic differences Thus, the abnormal in the neoplastic cell need not be a plus material (virus or agent) but a changed nucleoprotein complex This is in essence the theory of numerous investigators Malignancy in relation to organization and differentiation has been reviewed recently in this journal (5)

The transplantation experiments described demonstrate differences between normal F1 cells and neoplastic F1 cells In order to explain this difference, more knowledge is required of chromosomes of blood cells and of the assumed cytoplasmic reproductive cell determinants For such studies uniform populations of normal and neoplastic blood cells are now available, and the recent advances in histochemistry, electron microscopy, the introduction of tagged elements, etc might enable a more precise definition of the abnormal in the neoplastic cell than has hitherto been possible

## CLOSING COMMENTS

Contrary to general belief, control of a disease may either precede or follow knowledge of its cause and character Basic unknowns can be investigated irrespective of their logical sequence factors regulating normal hemopoiesis, the causation of leukemia, the fundamental change in leukemogenesis, the

character of the leukemic cell and the factors influencing its growth Each of these major problems can be resolved into a mosaic of minor questions Some of these and the facts gathered to answer them during the past few years have been reviewed. Solidity of facts gives strength to a theory Major discoveries have been announced on the basis of a single or few small experiments and left abandoned. Leukemia and leukemoid tissue reactions have often not been distinguished. Factors modifying the growth of grafts have been confused with those of the origin and growth of leukemic cells in their native host. Heredity, age, sex, nutrition and maternal influences are some of the ever present and often forgotten variables modifying, if not determining the action of an agent chosen for study In interpreting and analyzing experimental results, there is often a lack of integration with data of others and the generalization made is sometimes not warranted

The essence of the leukemic change appears as follows diverse extrinsic and genetic factors acting on normal hemopoietic cells result in the formation of an abnormal immature blood cell with a heightened growth force Thus, the leukemic cell, possesses individual characteristics which distinguish it from immature normal cells of its host and from other leukemic cells Its most important characteristics are failure of complete differentiation and ability to overcome the forces which restrain the growth of normal immature cells of its kind, so that it invades tissues and organs and ultimately kills its host It may remain at the site of origin or migrate around the body multiplying at favored sites The basic change is intrinsic in the cell since it is maintained in tissue cultures The change involves the enzymatic and metabolic capacities of the cell but no such single qualitative change is known which would identify a neoplastic blood cell The basic change may be due to *a*, an alteration of the reproductive material of the cells, either cytoplasmic or nuclear (the theory of somatic mutation), or *b*, to the presence of a selfperpetuating exogenous agent (virus theory in a restricted sense), or *c*, to an endogenous growth factor multiplying autocatalytically, causing abnormal differentiation

The origin of the somatic mutation theory is obscure It is associated with the name of Boveri but had many proponents The virus theory is supported by the work of Rous, Andrewes, Bittner etc (cf 97) and that of abnormal differentiation related to organizer effects is suggested by Loeb, Murphy, Needham, etc (cf 85) The current trend is for the integration of these theories as it seems that the frontiers existing between studies of heredity, development and infection are only technical and arbitrary (Darlington)

I gratefully acknowledge the extended support given to investigations in this Department by The Lady Tata Memorial Trust The International Cancer Research Foundation The Jane Coffin Childs Memorial Fund for Medical Research The Anna Fuller Fund, and by The Anonymous Donor who initiated these studies

## REFERENCES

(1) Abels J C F L Jones L F Craver and C P Rhoads Cancer Research 4: 149, 1944
(2) Barnes W A and R. K. Cole Cancer Research 1 90 1941
(3a) Barnes, W A and J Furth Am J Cancer 30 75 1937
(3b) Barnes W A. and J Furth Am J Path 16 457, 1940

(4) BARNES, W A AND I E SISMAN Am J Cancer 37 1, 1939
(5) BERRILL, N J Physiol Rev 23 101, 1943
(6a) BICHEL, J Ztschr f Krebsforsch 48 92, 1938
(6b) BICHEL, J Monograph of Univ Press of Aarhus, Denmark, 1939
(7a) BIELSCHOWSKY, F Brit J Exper Biol 25 1, 1944
(7b) BIELSCHOWSKY, F Ibid 25 90, 1944
(8a) BLAKEMORE, F J Comp Path and Therap 52 144, 1939
(8b) BLAKEMORE, F Proc Roy Soc Med 34 109, 1941
(9) BREEDIS, C J Exper Med 76 221, 1942
(10) BRUES, A M AND B B MARBLE Am J Cancer 37 45, 1939
(11) BURK, D , H SPRINCE, J M SPANGLER, M C BOON AND J FURTH J Nat Cancer Inst 3 249, 1942
(12a) BURK, D AND R J WINZLER In Vitamins and hormones *II*, 1944
(12b) BURK, D AND R J WINZLER Ann Rev Biochem 13 487, 1944
(13) CAMERON, G , C J KENSLER AND R CHAMBERS Cancer Research 4 495, 1944
(14) CHAMBERS, R , G CAMERON AND M J KOPAC Cancer Research 3 293, 1943
(15a) CLAUDE, A J Exper Med 80 19, 1944
(15b) CLAUDE, A Cold Spring Harbor Symposia on Quantitative Biology Cold Spring Harbor Long Island Biological Association 9 263, 1941
(16) CLAUDE, A AND F FULLAM J Exper Med 81 51, 1945
(17) CLAUDE, A AND J B MURPHY Physiol Rev 13 246, 1933
(18) COLE, R K AND J FURTH Cancer Research 1 957, 1941
(19) DARLINGTON, C D Nature 154 164, 1944
(20) DIXON, T F Nature 155 596, 1945
(21) DOLJANSKI, L AND M PIKOVSKI Cancer Research 2 626, 1942
(22a) DOUGHERTY, T F AND A WHITE Proc Soc Exper Biol and Med 53 132, 1943
(22b) DOUGHERTY, T F AND A WHITE Science 98 367, 1943
(23) DURAN-REYNALS, F Cancer Research 3 569, 1943
(24) ELLERMANN, V The leucosis of fowls and leucemia problems Gyldendal, London, 1921
(25) ENGELBRETH-HOLM, J Leukemia in animals Oliver and Boyd, Edinburgh, 1942
(26) ENGELBRETH-HOLM, J AND H ELTORM Acta path et microbiol Scand 20 346, 1943
(27) ENGELBRETH-HOLM, J AND O FREDERIKSON Acta path et microbiol Scand Suppl 37 145, 1938
(28) ERF, L A Am J Med Sc 203 529, 1942
(29) FARRIS, E J AND E H YEAKEL Am J Path 20 773, 1944
(30) FISCHER, A Virchow's Arch 310 395, 1943
(31) FLORY, C M , J FURTH, J A SAXTON, JR AND L REINER Cancer Research 3 729, 1943
(32) FORKNER, C E Leukemia and allied disorders MacMillan, New York, 1938
(33a) FURTH, J J Exper Med 69 13, 1939
(33b) FURTH, J Wisconsin University Press 106 125, 1939
(33c) FURTH, J AAAS Cancer Symposium, 1945 In print
(33d) FURTH, J Unpublished data
(33e) FURTH, J J Gerontology To be published
(34) FURTH, J AND W A BARNES Cancer Research 1 17, 1941
(35a) FURTH, J AND M C BOON Science 98 138, 1943
(35b) FURTH, J AND M C BOON AAAS Cancer Symposium, 1945, in print
(36) FURTH, J , M C BOON AND N KALISS Cancer Research 4 1, 1944
(37) FURTH, J , R K COLE AND M C BOON Cancer Research 2 280, 1942
(38) FURTH, J , H W FERRIS AND P REZNIKOFF J A M A 105 1824, 1935
(39) FURTH, J AND N KRUMDIECK Am J Path 16 449, 1940
(40) GATES, R R Human genetics In press
(41) GARDNER, W U , T F DOUGHERTY AND W L WILLIAMS Cancer Research 4 73, 1944

(42) Gardner, W U, A Kirschbaum and L C Strong Arch Path 29: 1 1940
(43) Gilmour M D J Path and Bact 45: 179 1937
(44) Gorer, P A J Path and Bact 54 51, 1942
(45) Gottschalk, R G Cancer Research 3: 649, 1943
(46) Grand, C G Proc Soc Exper Biol and Med 56 229, 1944
(47) Haddow, A Nature 154 194, 1944
(48) Hall V E Cancer Research 4 785 1944
(49) Heilman F R and E C Kendall Endocrinology 34: 416 1944
(50) Heinle R W, J T Wearn, D R Weir and F A Rose Ann Int Med 17: 902, 1942
(51a) Henshaw, P S J Nat Cancer Inst 4 513 1944
(51b) Henshaw P S Radiology 43 279 1944
(52) Henshaw, P S and J W Hawkins J Nat Cancer Inst 4 339, 1944
(53) Hirschmann, R R. R W Heinle and J T Wearn Proc Soc Exper Biol and Med 53: 5 1945
(54) Hutt F B, R K. Cole, M Ball, J H Buckner and R. F Ball Poultry Sc 23 396, 1944
(55) Israëls M C G J Path and Bact. 51 235 1940
(56) Jungherr E and W Landauer Conn (Storrs) Agric Exper Sta Bull 222: 5 1938
(57) Kabat E A J Immunol 47 513 1943
(58) Kaliss N and T Robertson Genetics 28 78 1943
(59) Kaplan H S and A Kirschbaum Proc Soc Exper Biol and Med 55 262 1944
(60a) Kirschbaum A Proc Soc Exper Biol and Med 55 147 1944
(60b) Kirschbaum A Yale J Biol and Med 17: 163 1944
(61) Kirschbaum A and H S Kaplan Science 100 360 1944
(62a) Kirschbaum A and L C Strong Am J Cancer 37: 400 1939
(62b) Kirschbaum A and L C Strong Cancer Research 1 785 1941
(62c) Kirschbaum A and L C Strong Proc Soc Exper Biol and Med 51: 404, 1942
(62d) Kirschbaum A and L C Strong Cancer Research 2 841 1942
(63) Law L W Cancer Research 4: 257, 1944
(64) Lawrence, J H Am J Roentgenol 43: 283 1942
(65) Lewis M R Am J Cancer 29 510, 1937
(66) Lewis, M R. and G B Mider J Nat Cancer Inst 2 115 1941
(67) Lewis W H and M R Lewis In cooperation in Research Carnegie Institute of Washington Publication 501: 369 1938
(68) Litt F A Kirschbaum and L C Strong Am J Cancer 34: 196 1938
(69) Loeb L Biological basis of individuality Thomas Springfield, 1944
(70) March H C Radiology 43: 275 1944
(71a) Marshak A J Gen Physiol 25: 275 1941
(71b) Marshak, A Radiology 39 621, 1942
(72) Maynéard W V and L D Parsons J Path and Bact 45 35 1937
(73a) Mercier L Compt rend Soc de biol 124: 403 1937
(73b) Mercier L Ibid 133 29 1940
(74) Miller, F R W A Hause and H W Jones Proc Soc Exper Biol and Med 50 115 1942
(75) Miller F R and D L Turner. Am J Med Sc 206: 146, 1943
(76) Morton J J and G B Mider Science 87: 327 1938
(77) Mottram J C Am J Cancer 30: 746 1937
(78a) Murphy, J B Monograph of Rockefeller Inst for Med Res no 21 1926
(78b) Murphy J B Cancer Research 4 622 1944
(79a) Murphy J B and E Sturm Science 98: 568 1943
(79b) Murphy J B and E Sturm Cancer Research 3 173 1943
(79c) Murphy, J B and E Sturm Science 99 303 1944
(80a) MacDowell, E C J Heredity 28 131, 1937

(80b) MacDowell, E C In Cooperation in Research Carnegie Institute of Washington Publication 501 353, 1938
(81a) MacDowell, E C, J S Potter and M J Taylor Proc Nat Acad Sci 25 416, 1939
(81b) MacDowell, E C, J S Potter and M J Taylor Cancer Research 5 65, 1945
(82) MacDowell, E C, J S Potter, M J Taylor and E N Ward Rec Genetics Soc America 9 160, 1940
(83) MacDowell, E C, J S Potter, J Victor, M J Taylor, T Laanes, E N Ward and M P Wintersteiner Carnegie Institute of Washington Year Book 36 51, 1937
(84a) McEndy, D P, M C Boon and J Furth J Nat Cancer Inst 3 227, 1942
(84b) McEndy, D P, M C Boon and J Furth Cancer Research 4 377, 1944
(85) Needham, J Biochemistry and morphogenesis Cambridge Univ Press, Cambridge, 1942
(86) Oberling, C The riddle of cancer Yale Univ Press, New Haven, 1944
(87) Olson, C Cancer Research 4 707, 1944
(88) Opie, E L Proc Inst Med Chicago 14 382, 1943
(89) Parks, A S Physiol Rev 25 203, 1945
(90) Parsons, L D J Path and Bact 54 321, 1942
(91) Paul, J T, L R Limarzi and L Seed Am J Med Sc 206 625, 1943
(92) Perla, D and J M Gottesman Proc Soc Exper Biol and Med 28 475, 1931
(93a) Pierce, M Arch Path 34 538, 1942
(93b) Pierce, M Am J Path 18 1127, 1942
(94) Potter, J S, J Victor and E N Ward Am J Path 19 239, 1943
(95) Potter, V R Science 101 609, 1945
(96) Richter, M N and E C MacDowell Physiol Rev 15 509, 1935
(97) Rous, P J A M A 122 573, 1943
(98) Saxton, J A, M C Boon and J Furth Cancer Research 4 401, 1944
(99) Schweitzer, M D and J Furth Am J Cancer 37 224, 1939
(100) Scott, K G Cancer Research 5 365, 1945
(101) Selye, H Brit J Exper Path 17 234, 1936
(102) Singher, H O, J C Abels, L F Craver and C P Rhoads Cancer Research 4 444, 1944
(103) Slye, M Am J Cancer 15 1361, 1931
(104) Stamer, S Acta path et microbiol Scand Suppl 47 1, 1943
(105) Stamer, S and J Engelbreth-Holm Acta path et microbiol Scand 20 360, 1943
(106) Stoerk, H C and T F Zucker Proc Soc Exper Biol and Med 56 151, 1944
(107) Sturm, E and J B Murphy Cancer Research 4 384, 1944
(108a) Tannenbaum, A Arch Path 30 509, 1940
(108b) Tannenbaum, A Cancer Research 2 460, 1942
(108c) Tannenbaum, A Cancer Research 4 678, 1944
(109) Thiersch, J B Australian J Exper Biol and Med Sc 22 57, 1944
(110) Turner, D L and F R Miller J Biol Chem 147 573, 1943
(111) Victor, J and J S Potter Brit J Exper Path 19 227, 1938
(112) Warren, C O Cancer Research 3 621, 1943
(113a) Warren, S Cancer Research 3 334, 1943
(113b) Warren, S Ibid 3 872, 1943
(114) Waters, N F Poultry Sc 24 259, 1945
(115) White, F R, J White, G B Mider, M G Kelly and W E Heston J Nat Cancer Inst 5 43, 1944
(116) White, J, G B Mider and W E Heston J Nat Cancer Inst 4 409, 1944
(117) Williams, W L Anat Rec suppl 1944
(118) Wilson, R H, F DeEds and A J Cox, Jr Cancer Research 1 595, 1941

# NEURAL MECHANISMS OF CUTANEOUS SENSE

GEO H BISHOP[1]

*Laboratory of Neurophysiology, Oscar Johnson Institute, Washington University Medical School, St Louis*

Following numerous recent reviews of the cutaneous senses, the present effort may appropriately emphasize a particular aspect of sensory function. The underlying concept will be that all sensory messages, on their passage through nerves from the periphery to the center, must consist of patterns of non-specific nerve impulses The consequent problem is then to correlate the pattern of the impulses with the sensation induced, considering the nerve impulses as the coin in terms of which we may evaluate sensation on the one hand, and stimulation on the other These patterns can vary only in limited respects to account for variations in sensation

First, different fibers, for instance large and small, may carry impulses of different energy value, i.e , of different denominations as coins The significance of this is little understood Second, any one fiber may fire at different frequencies, and for different durations, resulting in an accumulation or increase of sensory value in terms of temporal summation Third, fibers ending at different points of the skin or even at the same point may be activated together, at the same or at different frequencies, resulting in spatial summation of sensory values, sometimes, as might be expected, indistinguishable in sense from temporal summation No other variations would seem to be possible in the nerve-impulse patterns of sensory messages

Two other types of specialization of function however exist In the periphery the endings of the fibers are differently located, are differentially sensitive to different forms of energy, and have different properties appearing as adaptation, threshold, refractoriness, after-discharge, etc The character of the pattern of impulses set up by any external stimulus is largely determined by these factors Then centrally the different fibers impinge on different cells or areas of cortex, or make differential connections in some other characteristic manner, such that impulses however originated arriving over different fibers set up different sensory effects It is further probable that at the synapses along the fiber pathways to the cortex, differences exist characteristic of different groups of fibers or of sensory pathways, modifying the pattern from the periphery which is received at the cortex. In addition to these specific differences in connections, the sensory effect is further dependent on the state of excitability or of activity of the center, usually conceived of in terms such as mental or emotional attitude, state of attention, memory, etc It is also dependent on the state of the periphery determining the effects of a given stimulus on the pattern, for which terms of a corresponding elegance have not been elaborated At both center and periphery the state of excitability and the consequent magnitude and form of response may be further altered by current events, that is, by the stimulus itself at the periphery, and by

[1] Assisted by a grant for research in neurophysiology from the Rockefeller Foundation

the impulses arriving at the center The final and overall effect of all these factors is the sensory process

It will thus be obvious that skin sensation is more than skin-deep In fact the only portion pertaining directly to the skin of the extensive nervous machinery which performs a sensory act is the peripheral skin "sense" organ Even it determines only in a preliminary way what sensation is aroused by an external stimulus The term "cutaneous sensation" is appropriate only to suggest that a class of discriminatory or affective states of the central brain is potentially but not necessarily aroused by certain agents acting on the skin To these agents the skin is exposed by the incidental circumstance that it finds itself lying across the pathway of some physical force aimed at the organism in general Further, sensation is only one of the ways in which the organism reacts, for it also reacts to the same stimulus at various levels of the nervous system beside the sensory

Sensory pathways are in fact only a particular selection of the afferents to the central nervous system They consist actually only of those afferents the results of whose excitation may reach consciousness Sensation being registered in the brain, wherever along the pathway activation occurs, it is obviously more than the functioning even of these sensory afferents It is therefore appropriate to examine first the criteria by which "cutaneous sensation" may be differentiated out of the complex of nervous system behavior

*The concept of modality* The four modalities of cutaneous sense usually recognized, touch, pain, cool and warm, are differentiated on a dual basis, subjective and objective, and this has given rise to some confusion in the defining of sensory categories Moreover the flexibility of nervous system behavior leads to such variation in results of stimulation as to require rather arbitrary and perhaps artificial conditions of experimentation in the derivation of a formal classification Centrally, the ability exists to accurately differentiate subjectively, *at least* four sensations in simple experimental situations, and peripherally, at least four corresponding classes of endings in the skin can be inferred, if not ultimately demonstrated histologically, reacting typically but not exclusively to different and characteristic forms of energy It is a degree of correspondence between these subjective, peripheral and physical phenomena which gives meaning to the idea of modality All other sensations aroused by cutaneous stimuli can then be defined as summations of these four components, in various combinations and intensities Two difficulties arise in this connection

First, more than four types of endings are present, as judged by punctate stimulation of the skin at least, and second, the various stimulation of one point, and presumably of one type of ending, may arouse different sensations It would seem logically necessary for instance to include in the pain modality the rather pleasant sensation derived from scratching an itch, which must stimulate pain endings, and probably to assign itch itself to the result of a particular pattern of activation of pain endings Again the vibratory sense, although distinguishable from either touch or pressure, probably employs both pathways in a characteristic pattern of activation, and several distinguishable varieties of touch following point stimulation of skin are included in one modality merely because they seem

to have, subjectively, a common quality Still other sensations are more obviously the result of a more complex pattern of multiple stimulation, wetness, smoothness, etc The selection of four fundamental modalities for the classification of cutaneous sensations is thus a compromise between simplicity and confusion, and between specificity of stimulation and flexibility of central interpretation

One may then define pragmatically the modality of warmth as that sensory process set off with least energy when heat only is applied as a stimulus to the normal or average subject, over a considerable skin surface Cool can be defined as the sensation aroused from the stimulation by an adequate stimulus of certain discrete points usually occurring in groups deep in the skin, and which can be identified as those responding preferentially when lowered temperature is the only stimulus Pain as a cutaneous modality is the response to any stimulus at certain discrete points in the skin, characterized normally by an avoidance or withdrawal reaction if the stimulus is sufficiently intense, and arousing a central sensation which is its own definition Below this intensity of stimulation these sense organs arouse a contact or prick sensation which is not painful subjectively In this the pain system appears to differ from those of warm, cool and touch. Its normal stimuli are mechanical distortion of the skin, and a more intense change of temperature than is required for temperature sense It is peculiarly liable to increase of irritability under the influence of inflammation The modality of touch then includes all other senses now known to be aroused by simple stimulation of discrete skin loci or nerve endings, a contact sense normally activated only by mechanical distortion

A workable classification of cutaneous sensation thus requires a set of criteria which satisfy various conditions, recognition subjectively of discrete and characteristic sensations, specific nerve mechanisms in the periphery, and characteristic and preferential forms of stimulation A further examination of the characteristics of the pathways between periphery and brain for each sense insofar as at present known may throw further light on the processes by which any stimulus pattern induces its sensory response This would seem to be an important if neglected aspect of sensation It will appear that in many respects the whole pathway for a given sense is characteristic of that modality, in the sense that its mode of action, synaptic connections and structure contribute to the pattern of nerve impulses in which the effects of the peripheral stimulus are recorded at the central level

One of the chief supports for the concept of modalities has been the presumed specificity of skin endings for different forms of energy Yet von Frey (1896) recognized that the relative thresholds for pain and for touch depend on the area of the contact of the stimulating object, and the structure of the skin itself, and the position of the nerve endings within it (Watterston, 1933, Bishop, 1944), probably play as important a part in selective stimulation as does the selective irritability of the nerve endings Hardy et al (1939) find radiant heat a most effective stimulus to pain endings, paradoxical stimulation of cold spots is a familiar phenomenon, all the sense organs except warm spots are excitable elec-

trically (Bishop, 1943) and with relative thresholds for touch and for pain opposite to those disclosed by mechanical stimulation, and Nafe (1942) considers changes in vessel size the immediate stimulus in the response of temperature endings This author emphasizes the possibility that the nerve endings themselves may be non-discriminative as to forms of energy applied, a possibility previously considered by Bronk (1934) What degree of specificity nerve terminals themselves are capable of is in doubt, and the overall selectivity may best be considered as a function of many factors, position in the skin, encapsulation by non-nervous envelopes, including the dermal tissue itself as a "capsule" or medium through which external agents affect "free" nerve terminals, density or stiffness of overlying epidermis, affected by sweating, etc , and not least, the way in which the energy is applied in customary experience When variations in these factors are minimized or controlled experimentally the sensation elicited is liable to depart from the casual experience which determines our concepts of sensations, and further complications arise A certain amount of training of the subject is in fact necessary to obtain reliable data under many experimental conditions It can then be said that only by taking the "sense organ" as a complex of the sensory nerve terminal, its surroundings in the skin, and the circumstances under which it is usually stimulated, does a high degree of specificity with respect to energy selection result

*The pathway to the higher centers* Between the sense organ sensitive to a specific type of energy and the area of the brain where its sensory message is projected extends the fiber pathway, interrupted at various levels of synaptic junctions This pathway may be characterized in several ways, other than by its central and peripheral terminations One is the size of fiber of which it consists, at least in peripheral nerves, together with the fiber properties which go with size A second is the segregation of afferents into functional groups in the spinal cord A third is the type of response other than sensation,—reflexes for instance, —induced by peripheral stimulation A fourth may be the degree of interconnection between parallel pathways of a given sense at synaptic regions, such as would permit spatial summation, for example In a sense the reverse of this, point discrimination or recognition of position on the body surface, might be a function of the degree of separateness of individual pathways, although to what extent subcortical neurones are involved in this, and to what extent it depends on interpretation of stimulus pattern at the higher centers is unknown Tower (1943) has offered a theory of localization involving central recognition of the pattern of peripheral ending activation, rather than a simple one-tract pathway from periphery to center, and Lorente de Nó's concept of "partially shifted overlap" (1934), consistent with many observations on the structure and functioning of neurones, presents a picture of a given axon's terminals impinging on the dendrites of several neurones, the selection of the post-synaptic element to be activated depending on the pattern of activity of the pre-synaptics In the geniculate nucleus of the optic tract a sensory pathway capable surely of the highest discrimination shows a pre- to post-synaptic relation corresponding to this concept (O'Leary, 1940) and certainly not to a one-to-one relation There is no reason

to believe that pre-to-post connections at the spinal cord level are any more unique, and the differences between the various senses with respect to discrimination of locus on the one hand and spatial summation on the other have at present no final experimental explanation

*The constituent fibers* of a skin nerve when classified according to size fall into several groups whose maxima are distinct but whose margins are not (Gasser and Erlanger, 1927) Since threshold as well as conduction rate vary with size, the action currents after conduction segregate into corresponding waves, one for each size group, and the strength of stimulus can be so manipulated as to stimulate fibers only above a selected size Block by pressure affects large fibers preferentially, and a combination of pressure block and limited stimulus strength will serve to activate a selected band of fiber sizes by eliminating from stimulation both larger and smaller fibers than those selected

Both in (nanesthetised animals and in man (Heinbecker, O'Leary and Bishop, 1933, Heinbecker, Bishop and O'Leary, 1933) procedures of selective activation of skin nerve branches demonstrate that the larger fibers from the skin serve touch only, at any number and frequency of stimulation, while a fairly distinct group of medium-sized fibers (the delta group, 6 to 3 mu in diameter) include pain fibers. One cannot say from this evidence that smaller fibers, in the delta range for instance, do not carry touch or pressure, and in fact there is evidence that they do, and to be sure the fact that two kinds of touch are recognizable in the skin, that from hair follicles and that from areas of skin between them, leaves the possibility at least that even within the one modality of touch a differentiation with respect to size exists Other evidence, from stimulation of individual pain and touch spots of the skin, indicates at any rate that in the delta range also touch fibers if present do not mediate pain Just below the pain size and probably overlapping its distribution in the same group are fibers serving temperature senses (Heinbecker, Bishop and O'Leary, 1934) Still smaller fibers, the C or non myelinated group, can be demonstrated also to cause pain on stimulation (Clark, Hughes and Gasser, 1935) What other senses this group may mediate is not known at present

Not all the fibers in each size range are known to be sure to serve these respective modalities, or even to serve sensation at all, further the margins of the size groups are not sharply discrete, nor do the experimental procedures employable separate too precisely according to size However the demonstration that fibers above a certain size and threshold carry touch but no pain, that fibers just below this in size carry the pricking pain of the skin, and that the largest pain fibers are approximately at the head of the delta group, can be made clearly in conscious human subjects (Heinbecker, Bishop and O'Leary, 1934) Further, differential anesthesia of nerves in man (Heinbecker, Bishop and O'Leary, 1933) indicates that the temperature senses are mediated by fibers in the delta group in general smaller than, but overlapping the pain fiber size Temperature has not been excluded from the C group Not only the exclusion of pain from overstimulation of touch fibers, but the convenient and obvious activation of pain endings by heat (Hardy et al., l c ) suggests that "hot" pain is also mediated by pain fibers and

not by temperature fibers, that is hot or burning pain is a composite of warmth and pain (Pathological or "burning pain" of the thalamic syndrome is not necessarily so accounted for) Pain from excessive cold may be not a skin sense, but arise from activation of deeper pain endings, or from both superficial and deep (Wolff and Hardy, 1943), there is no evidence that it arises from excessive stimulation of fibers mediating cold itself It is perhaps noteworthy that pain from muscle nerves and from stimulation of the sympathetic trunks (visceral pain?) in animals arises also from activation of the same sized fibers as in the skin

On the basis of animal experiments this concept has been questioned, particularly that pain fibers were confined to the delta and C groups (Gasser, 1943, and others there cited) For instance, stimuli known to be injurious or to arouse pain activate fibers whose conduction rates extend over the whole size range, although it would seem not necessarily to follow that all fibers so stimulated contribute to the pain, without a more discriminating test of their separate functions Also, the fact that pain is mediated by two groups so far apart as the delta and C might raise doubts as to the significance of size Nafe (l c) arguing for a theory of non-specificity of nerve fibers cavalierly dismisses the idea of specific size group correlation with the statement that the attempt to identify pain fibers by size has failed, and decides that the overstimulation of touch mechanisms causes pain An enthusiastic reinterpretation of the evidence on human nerve stimulation permits him to draw conclusions the diametric opposite of those of the experimenters he cites (Heinbecker, Bishop and O'Leary, 1933) Other discrepancies reported in the literature on fiber size and function may serve as the basis for legitimate doubts as to the precision of the correlation until these discrepancies shall have been disposed of On the other hand, the absence of an alpha group in skin nerves and its presence in nerves to muscle provides the experimenter with an ace cold, in that no cutaneous sense can be mediated by fibers as large as the group that carries impulses for the proprioceptive sense of muscle Stated with due qualifications the conclusions here reached are not irreconcilable with the position of Gasser (l c) sometimes cited without his own qualifying statements as the extreme proponent of the opposite view Beyond a considerable body of accepted findings, divergent inferences as to an ultimate solution are as proper as they are tentative

From such evidence then it is permissible to infer that the pathways of sense, at least in their course through peripheral nerves, are indeed mediated by fibers of characteristic size ranges, though the ranges for the different modalities overlap at their boundaries No conclusive reason can be assigned for this distribution, perhaps the higher conduction rate of large fibers makes them more suitable for the more discriminatory senses, touch in the skin and the proprioceptive senses from muscle, etc Or fast impulses may set the stage for slower ones at synaptic levels (Gasser, 1935) The occurrence of two bands, delta and C fibers, in the nerves' size spectrum which mediate pain may prove to be the salubrious exception, and mean that two varieties of pain are mediated, for which there is some evidence

It was noted by Goldscheider (1881) that response to pinprick seemed to be

double, an immediate prick followed by a slightly delayed aching pain Von Frey in repeating these experiments (l c ) claimed that only the delayed pain arose from stimulation of pain spots in the skin in certain loci where touch was not aroused, and he diagnosed the first sensation as touch, interpreted as pain when followed by the second phase of the double response The present writer confirms von Frey, and further confirms Lewis and Pochin (1937) that by electrical stimulation of pain spots only one sensation is induced, touch and presumably the pain from C fibers requiring a higher strength of stimulation by electricity than delta pain (Bishop, 1943) However, a double pain sensation has been reported repeatedly and Lewis and Pochin assign the second phase to the slower conduction of impulses in C fibers, assuming both to be activated at the same instant The change in the character of sensation as the pain from a severe prick develops, and measurements of the reaction times from points at different distances from the cord have led to the suggestion that ordinary prick is mediated by the faster delta fibers, aching pain by the slower The same aching pain can, however, be elicited as the sensory effect of repetitive electrical stimulation, at a strength such that one shock alone calls forth only bright prick Unfortunately, all the sensory fibers lose their myelin sheaths as they approach the epithelium, preventing histological identification of point innervation in this respect Another possibility exists, that non myelinated fibers serve that pain which is difficult to elicit in normal subcutaneous or visceral tissues, but which is rendered extremely irritable in inflammatory reactions Lewis and Pochin point out that a non painful interval between early and delayed pain must indicate a discontinuity of pain fiber distribution between myelinated and nonmyelinated pain groups (larger delta and C), consistent with the known interval in conduction rate

Until less inferential conclusions can be drawn from more critical evidence, the *differences* in *sensory responses* to delta and to C fibers cannot be said to have been unequivocally demonstrated Their reflexes do differ significantly (see below) In view of the necessity that C pain must be delayed compared to delta pain if both are elicited at once, it may be suggested that there are two phenomena here confused, an *apparent* double pain when touch and delta pain fibers are stimulated together, and a true double pain from delta and C simultaneous activation, and that their respective elicitation may be a function of intensity of stimulation

*Sensory pathways at the cord* level segregate roughly according to modality, and at least on a functional basis Those mediating touch discrimination pass with the proprioceptive discriminative paths up the dorsal column to synapse in the medulla, and their reflex collaterals synapse chiefly with cells in the dorsal and lateral margins of the grey horn Pain and temperature fibers enter the tract of Lissauer and leave it near the level of entry to synapse in the substantia gelatinosa From here the post-synaptic fibers cross to the contralateral spinothalamic tract where temperature, pain and a residual touch localization further segregate dorsal to ventral in the order named The segregation of the dorsal column discriminatory fibers from the rest at the root entry zone into the medial and lateral divisions of Ranson (1913) separates the large and small fibers of the sensory

myelinated and non-myelinated, to make the functional cord segregation thoroughly consistent with the assignment of modalities to their respective fiber sizes in the peripheral nerves Beyond the first synapses the sizes of the post-synaptic fibers have not been measured, and admixture with the ascending and descending pathways serving other functions might render this difficult However, the velocities of the dorsal column continuations of the larger fibers of the saphenous are slow and of smaller size than in the peripheral nerves (Gasser, 1943) which suggests that the value of large size and fast conduction pertains to cord functions rather than to those at higher levels

One can only speculate as to the significance of this segregation at the cord level, although its correspondence with the classification into modalities on other grounds offers convincing support for the significance of that concept At the pons and midbrain level these separated pathways, together with corresponding fibers from the cranial nerves which have segregated similarly, rejoin to form the sensory lemniscus passing to the thalamus So far as is known, touch pain and temperature do not have discrete projection areas in the cortex, although they may have in the thalamus The discriminatory sensory components are the ones making connection by collaterals with the cerebellum, via cells of the cord adjacent to the dorsal columns, and this may be a factor involved in their spinal segregation From the facts, however, that the segregation takes place at the level of entry, and that reflex connections are made near that level, and that the reflex responses to stimulation of different modalities are quite different, one might infer that the cord segregation served primarily cord functions, and a search for its rationale might be made in those aspects of cord organization more primitive than is sensory projection to the higher centers This would be consistent with the view that higher pathways for pain, touch discrimination, cerebellar projection, etc , were added at different phylogenetic stages to a fundamental cord pattern of afferents serving basically spinal cord activity

*Differences in reflex effects* of stimulating fibers of different size, and hence of modality, seem to support this view that the spinal segregation is essentially spinal in significance, a view with which the reduction in size of large fibers after they send collaterals to the cord neurones is consistent Whatever may be the utility of consciousness in organizing body activity, its increasing dominance over body reactions must have utilized the pattern of organization of animals with less elaborate cortices The function of the brain as a physiological device is to integrate the activities of the lower centers for more complex or more "purposeful" activity The addition of "voluntary" activity on the motor side, and of conscious sensation on the afferent side to provide a motivation for this activity, is only a further elaboration of the fundamental plan of activation of motor organs in response to stimulation of sensory endings by way of a central synaptic region of the cord segment From this point of view sensation itself is an incidental if effective refinement, the apical efflorescence on the afferent tree whose lower branches still bear most of the fruit The preoccupation of the most self-conscious of animals with their own emotions to the extent of narcissism has unhappily led to the isolation of "sensory" physiology from the field of response to stimulation

While it is true that in certain respects the ultimate as well as the most sensitive and significant criterion available to the physiologist is the response in consciousness of the human subject, this event in consciousness is only one of a group of response activities in general consistent with each other and fundamentally related It is perhaps not beyond the bounds of rational analysis to speak of modalities of reflex, as of sensory activity

This point of view has not dominated the field of reflex physiology, with the result that evidence to elaborate it is sketchy and often incidental. It is generally recognized, however, that pain in particular is peculiarly reflexogenic to muscles, a realization facilitated no doubt by the fact that the anesthetic employed in many animal experiments suppresses effectively the reflex response to any stimulus less drastic Dilatation of the pupil, respiratory and circulatory increases, even more than skeletal muscle contraction are specific enough to be taken conventionally as signs of painful stimulation even under anesthesia Many reflexes from skin afferents are called forth by touch, and can be demonstrated by electrical stimulation of afferent nerves in lightly anesthetized animals at a strength of shock which the nerve record shows to involve only fibers larger than delta (Bishop and Heinbecker, 1935) Not too much is known concerning reflexes to temperature stimuli below threshold for pain, but the familiar changes in skin blood supply and sweating are obviously reflex. Stimulation of delta and of C fibers of the saphenous separated by differential block (Bishop and Heinbecker, l. c ) gives a suggestive picture of different patterns of reflex responses With slowly repetitive shocks to delta fibers, each stimulus calls forth a twitch of the gastrocnemius, each twitch followed by an after-discharge if frequency or strength is sufficient This is in effect a "flicker" phenomenon, and as the effectiveness of stimulation is increased, fusion of individual responses takes place by increase of after-discharge to a sustained muscle contraction When C fibers only are stimulated, no twitches are obtained, the response to single effective stimuli being a scattered discharge of muscle elements Fusion to a steady contraction takes place at a much lower frequency, and with no abrupt contractions even approaching a twitch Granting that temporal dispersion in the slowly conducting C fibers may play a part in this phenomenon, one is tempted to compare these two muscle reflex patterns to the pattern of central activity that must underly prick and ache, respectively

To some extent, then, the excitation of each modality of sense also excites reflexes equally characteristic of that modality, and equally appropriate for adjusting the animal to a changing environment That two kinds of pain mechanisms even, delta and C, have different characteristics is supported by the effects of anesthesia on their central connections.

When dial is employed, for instance, stimulation of delta pain is relatively ineffective as compared to stimulation of C fibers (Clark, Hughes and Gasser, l c ) judged by reflex responses in cats When ether is administered C fiber reflex responses are entirely suppressed at an anesthetic level permitting brisk muscle

in animals may depend on the condition of the animal much as do conscious pain sensations in the human subject

A feature of this situation not sufficiently considered is the existence of non-painful "pain", which in casual experience must involve the contribution to many complex and non-painful sensations of components assignable technically to the pain modality Aside from the fact that persistent electrical stimulation of pain spots below threshold for pain induces itch, the scratch which follows, not to say pursues the itch, whatever else it accomplishes must stimulate also pain endings, in the paradoxical suppression of a phenomenon in its own modality Once the threshold, not of pain reaction, but of the perception of the minimal reaction of pain endings is recognized, the low threshold of this response permits participation of pain endings in any contact more drastic than the blandest touch Brushing the scalp, mopping the brow, vigorous rubbing and especially mild scraping of any surface and firm manipulation of tools, instruments, rough surfaces, etc , must constantly activate pain endings, which participate in discrimination, information and control of environmental objects far short of that activity inducing the protective reaction or the apprehensive emotion usually associated with pain

*The activity of skin sensory endings* Following the classical experiments of Blix, Goldscheider and von Frey, the study of skin endings was for a period directed largely to the location and histological identification of specific sense organs A study by von Frey (1896) of the difference in the character of skin distortion which activated preferentially touch and pain endings led him to assign touch stimulation to simple pressure distortion, pain stimulation to hydrostatic pressure differentials due secondarily to distortion A mathematical formulation of the supposed stresses accounted for the differences in actual thresholds Many subsequent experiments varying pressure and area of the stimulus object added little to the understanding of the essential character of sensory excitation The finding of specific selectivity of peripheral endings was complemented by Mueller's doctrine of specific nerve energies that however a sensory pathway was excited, its activity aroused the sensation characteristic of its central apparatus

With the development of electrical recording of nerve impulses, and Adrian and Bronk's (1928) technique for recording from single sensory fibers, a third factor became apparent, different sense organs activate their nerve fibers to different patterns of responses, each appropriate to the function which the given structure serves at the reflex or sensory level The chief differences in pattern involved may be summarized under the terms *adaptation* and *after-discharge,* a falling off of frequency under a constant stimulus, and a continuation of activity after the stimulus is removed They seem to be to some degree independent variables of sense organs

How these properties are determined at sensory endings is unknown With the exception of the simple retina of Limulus (Hartline and Graham, 1932) and by analogy perhaps the retinogram of higher forms, no response of any sense organ has been directly recorded, at least if one presumes that the sensory ending differs in action from the fiber it activates Cogent arguments have been presented that

it does differ (Bronk, 1936) This is demonstrable in the Limulus eye in that a long-lasting slow wave from the retinal cell body is accompanied by a train of typically brief impulses in the nerve fiber activated Otherwise the only information we have is derived from the temporal pattern of impulses in a sensory fiber when its ending is stimulated For instance, since responses in tactile fibers may follow the stimulus frequency applied to the skin to as high a rate as the fiber itself can be stimulated, Adrian, Cattell and Hoagland (1931) conclude that the tactile ending must have a refractory period as brief as that of its nerve fiber But whether this really means that the sense ending itself responds repeatedly at such a rate, or merely that each stimulus raises its persisting excitation to the point where it discharges its fiber, is not so certain Other evidence might suggest that some sense organs at least do not respond in an all-or none manner, but rather undergo a more persistent and graded excitation which activates the fiber periodically as a steady galvanic current does, at a frequency depending on the strength of the stimulus and decreasing with adaptation of the fiber to the stimulus

The nature of adaptation, of sense organ, nerve fiber or central synapse, is not clear, it is exemplified in the relative ineffectiveness of slowly increasing stimuli, and in the fact that a change in intensity of any stimulus is more effective than a steady state, and appears to be a property of all irritable tissues to all stimuli However, adaptation in excitable tissues can be materially modified In nerve the deprivation of calcium reduces it, at the same time increasing the irritability, until the fiber may respond spontaneously Treated with oxalate, frog touch endings, normally rapidly adapting, adapt more slowly, like normal pressure endings (Talaat, 1933) and finally become spontaneously and repetitively active, when adaptation is either abolished or effectively masked. It is possible that the effect of inflammation on pain response may involve changes in adaptation On the other hand as the "sense organ" should perhaps be considered as the whole complex of tissue surrounding the nerve ending which conditions the transformation of applied energy into nerve impulses, (supra) the mechanical properties of tissues undergoing distortion or physical stress will introduce a factor appearing as adaptation For instance, Nafe and Wagoner (1941) find that stimulation of contact endings parallels motion of the tissue under the stimulus, and conclude that adaptation consists of the gradual decrease of motion as resistance to further deformation balances the distorting force Adaptation is thus assigned to the non nervous tissue surrounding the nerve ending Although it is common experience that adaptation to adequate stimuli may not be complete even after an indefinite time for mechanical adjustment to applied energy, and that adaptation takes place to stimuli involving no motion in tissues, it is still possible that any factor inducing a decrement in effectiveness either of stimulus intensity or of fiber irritability will appear as adaptation under otherwise constant conditions

In terms of nerve fiber response, the characteristic pattern of a number of sense organs has been described, and Bronk (1934) has discussed the correspondence of these patterns to the type of sensation or reflex response on the one hand, and to

receptors of the lungs (Adrian, 1933) and of the carotid sinus (Bronk and Stella, 1935) adapt only slightly to persistent stretch, and show slight after-discharge Muscle stretch receptors (Bronk, 1929) adapt but little, and these and the receptors of the carotid sinus actually exhibit what might be called negative after-discharge, a silent period accompanying reduction of tension, which is followed by a reduced rate corresponding to the new tension Pain receptors (Tower, 1940, Hogg, 1935) adapt but little or even show the opposite phenomenon, inertia, indicated by a progressively increasing response during constant stimulation, and they show a persistent after-discharge Tactile endings adapt with extreme rapidity and completeness (Adrian, Cattell and Hoagland, 1931) without any after-discharge, although removal of the steady stimulus acts as a second stimulus Pressure endings in the skin or subcutaneous tissues adapt much less than do touch endings, and their after-discharge has not been specifically reported In the optic (Hartline, 1938) and auditory nerves (Galambos, 1944) an array of fibers is found which if classified in terms of adaptation and after-discharge not only covers the gamut of sense-organ combinations, but raises the question of the significance of these terms Some fibers show prompt adaptation, with no response at cessation of stimulation, some respond at cessation as well, others show but slight adaptation, or show a relatively silent period following an initial burst, followed again by a higher discharge, still others fail to respond during a stimulus but respond only at its termination Some of these phenomena may be complicated by the fact that the impulses are recorded postsynaptically to the sensory ganglion cells, which suggests that further investigation at the cord level of cutaneous sensory pathways might be profitable, in elucidating the modification of impulse pattern along the pathway from periphery to cortex In other words, adaptation and after-discharge may occur centrally to further modify the message to the center initiated by a given peripheral stimulus, compromising somewhat the interpretation of sense organ action from the report of the course of sensory experience

Still these differences in pattern of response of sensory endings, plus the modification of pattern at synaptic regions, must largely determine the form of the message received at the conscious level from a given pattern of stimulation Granted that the sense organ is initially selective with respect to different forms of energy, to what extent is its response, however stimulated, also characteristic of the modality it serves?

For pain and contact senses, obvious correlations exist between sense organ action and the type of information to be conveyed to the center (Adrian, 1928, Bronk, 1934) A threat of danger mediated by pain endings would be misleading if the sense organ responding ceased to act before the stimulus were disposed of, but the vibratory sense served by touch would be ineffective unless its sensory receptor were "dead beat", that is, promptly adaptive and without after-discharge Pressure and tension endings, non-adaptive and extremely sensitive to changes in stress in terms of frequency of response, follow persistently and flexibly any changes in their environment that affect them at all Concerning the temperature senses less is known in this respect, since animal experiments so

far dealing with these probably included responses of pain endings as well as of temperature endings to heat, but it is safe to anticipate a similar correlation

It may be noted that within the one "touch" modality, two types of endings, selective for two different aspects of contact, touch and pressure, transmit to consciousness two characteristically different types of pattern from a given pattern of stimulus Within the pain modality a further differentiation is evident, in which one type of ending induces different qualities of sensation by means of impulse patterns imposed on it by different stimuli While the different varieties of touch sensation, simple contact, moving contact or rubbing, repetitive contact or vibration, etc , may be readily recognized to have the common factor of bland touch, activation of "pain" endings runs a gamut of sensations from an indifferent contact sense through itch, prick, bright pain and ache (Bishop, 1948) To verify the assignment of all these sensations to one and the same type of ending electrical stimulation is useful for several reasons First the threshold for pain to electrical shocks is lower than for any other modality of skin sense Thus, if itch, for instance, is not induced by stimulation of pain endings, at least it is not assignable to touch, pressure or temperature endings Further the precision with which brief shocks can be manipulated into patterns of repetitive stimuli, and particularly the possibility of applying and removing them instantly, permits a precise knowledge of the applied stimulus for comparison with the response While it is not feasible to record directly from nerve fibers in conscious subjects, knowledge gained from animal experiments permits the pattern of nerve response to be inferred from the stimulus, to be then compared with the sensation induced It is then not impossible to infer from the sensory responses to specific patterns of stimulation the more complex patterns of response corresponding to more casual contact with the environment

Within these conditions, a persistent and annoying itch quite similar to that of an insect bite, is set up by rapid repetitive stimuli, each shock so weak that summation of the effects of several is required to produce any sensation at all It has a long after-effect, presumably due to the after-discharge characteristic of pain endings, which is promptly allayed by rubbing or scratching From the type of stimulus which induces itch, one may infer that the relief of itch by scratching is brought about by breaking up the monotony of nerve fiber response inducing the itch, not by abolishing the activity Itch also follows sharp prick as an after-effect of single stronger shocks To weaker single shocks, bright prick without itch is elicited, repetitive shocks as slow as 5 per second show pronounced summation, the overall sensation mounting over a period of one-half to one second, punctuated by increasingly sharp stabbing prick Above this range of strength prick shades gradually into pain, and pain that is received without aversion into pain to which the hardiest subject will finally protest In these various gradations frequency of repetition and intensity of shocks are equally effective in determining the amount and quality of sensation, indicating the effectiveness of both spatial and temporal summation, and of the summation of the after-discharge of one volley with the response to the next Adjacent pain spots also summate effectively In this sequence of sensations, foreknowledge of the

stimulus pattern permits a further qualification of the character of sensory discharge corresponding to each sensation

The threshold contact sense of the pain modality is merely the minimum of any activation which is able to break through into consciousness, too limited in amount to be discriminated Itch corresponds to a slow but persisting and uniform frequency of fiber response, which even follows the sharp prick of a single strong shock by reason of the gradual falling off of the after-discharge, approaching a steady frequency Prick and pricking pain differ from each other only by intensity of stimulation, therefore in number of fibers activated and the frequency of their response, that is, in the total number of responses per unit time arriving at consciousness from a given locus Aching pain differs from pricking pain apparently by reason of duration, that is, by the degree of central summation over time, of strong stimulation Its characteristic tone is not induced by one shock, however strong, nor by any number of shocks if weak Only myelinated or delta pain has so far been analyzed in this respect

While the pain modality exhibits perhaps the greatest qualitative range of sensation, touch is notoriously discriminative of the character of the stimulus object (smooth, uneven, moving, etc ) even when other modalities can be excluded from stimulation Skin tickle is apparently a form of touch as itch is of pain It seems to require an intermittent stimulus, or successive stimulation of adjacent points (movement) and stimulation below a certain intensity It can be faintly elicited by a hair attached to a tuning fork on one touch spot on the palm of the hand at an intensity below threshold for pain spots, but is increased by moving the vibrating hair across successive touch areas A wisp of cotton moved lightly over the upper lip can cause excruciating tickle, which is not obtained, however, if the pressure is increased, nor is it obtained by the lightest pressure from a mass of cotton that stimulates a larger area of the lip at once, even by a moving contact The temperature senses are probably less flexible in this sense, although their complex summation with touch and pain to give wet, hot, clammy, etc , are obvious

It will be apparent that these gradations in sensation within one modality connote most effective summation, spatial and temporal, at the conscious level of the effects of individual nerve impulses In contrast to this, Wolff has been quoted in the literature to the effect that pain is not summated Wolff's careful and definitive statement, however, (1943) is that "*beyond a minimal limit* in the normally innervated skin the *threshold* for pain was dependent alone upon the strength of the stimulus and not on the number of sense organs stimulated although the total distress to the individual is dependent both upon the intensity of the stimulus and the size of the painful area " (Italics ours ) Further he notes that in nerve lesions reducing the density of pain endings even the threshold for pain is increased This is to state that the *perceptual* threshold of first prick from a few endings is the same as that from many, provided that those few endings are normally concentrated, but that the *reaction* to pain depends upon the number of endings stimulated, as well as upon the number and frequency of discharges from each, that is, it is a function of the total number of impulses

per unit time Since this *reaction* to pain is to the casual sufferer pain itself, to quote without technical qualification that pain is not summated conveys the opposite of Wolff's findings as well as of his conclusions Wolff's statements apply further only to spatial summation, and the fact that his threshold response is measured at the end of a significant period of stimulation certainly does not exclude temporal summation, although it does not necessarily indicate it

That the experience of pain is about the most responsive to summation, especially to temporal summation, of any of the cutaneous senses, at least more so than touch, would epitomize common experience, and experimental stimulation of individual pain spots confirms this It reveals further a peculiar relationship between summation of intensity or of effect of sensation, and two-point discrimination for pain (Bishop, 44a) in that one tends to exclude the other in consciousness This is consistent with the fact that pain is both a discriminating and an affective sensation, being localized almost as well as touch, while capable of arousing an emotional effect more vigorous than that of any other sensation Consciousness appears to be to some degree limited to a choice between discrimination of locus and evaluation of quantity, and that choice is conditioned by peripheral as well as by central factors, that is, by the spatial and temporal pattern of impulses reaching consciousness as determined by the pattern of stimulation

Discrimination in general, of locus or of intensity, is poor near the threshold of stimulation for any sense Above threshold of perception, increase of central effect can be a function of at least three variables in the periphery, increase in the number and frequency of impulses from a given sensory ending or group of them corresponding to temporal summation, increase in the number of endings activated within a given modality, comprising spatial summation, and changes in relative degrees of activity of different modalities, or the overall pattern Increases in total stimulation of any pattern may have both quantitative and qualitative effects. Moreover, since the stimulation of a single ending, or even of endings of a single nerve fiber, is usually accomplished only under highly technical conditions, "pure" sensations, not to say simple stimulus patterns, are the exception in ordinary experience Slightly above an intensity suitable for touch, pain endings are liable to be stimulated below threshold for pain, and thus contribute to contact sense Fusion of contact and temperature senses is so usual as easily to escape notice, the sensation being given a special designation as wet, slimy, etc., or the contact aspect is ignored, although it contributes to the quality of the temperature sense Similarly touch, pressure and muscle sense are compounded in the estimation of weight or force In these complex patterns each aspect of the stimulus serves as a clue to the interpretation of the others in evaluating the external object or agency furnishing the stimulus

Aside from the associated emotional effect, the useful perception of sensation involves discrimination of intensity, quality or space Granted that intensity is obviously a function of frequency or of total number of impulses, and that modality (quality) is a function primarily of specific central connections, however complicated by concomitant circumstances central or peripheral, the question

remains, what aspect of the sensory pattern permits discrimination in space The naïve inference that spatial localization could be accounted for by direct spatial projection of the periphery on the cortex, with separate point-to-point connections by means of simple end-to-end chains of neurones has met with too many difficulties to be enumerated here, but enough to demand a more complicated explanation, and perhaps an explanation of more than *spatial* perception It must be granted that some degree of localization of the periphery on the cortex is indicated by the separate projection areas of the special senses, by the representation of successive regions of the body along the central sulcus, and particularly by the reproduction within the visual area of the geography of the retina Work on the peripheral distribution of sensory endings (reviewed by Tower, 1943) reveals that spatial localization, two-point discrimination, etc , is more precise than can be accounted for by the discrete distribution of separate fiber endings over the body surface, and in fact reveals that wide overlapping takes place in the ramifications of individual fibers What is known of the complexity of interconnections between parallel fiber pathways at synaptic regions, and which may be inferred to be especially pronounced at the cortical level, adds to the same difficulty Starting apparently with a basis for crude localization in region-to-region, not to say point-to-point projection of periphery onto center, the brain has elaborated the fineness of its discrimination beyond the limits which the degree of specificity of anatomical connections would permit, and without a corresponding increase in the detail of such projections Without attacking the problem of how consciousness perceives the pattern of activity of the brain, which must still be left in the realm of psychology, inferences are in order as to what patterns of activity reach the brain, corresponding to the perceptual acts of which it is capable

Such inferences have been made by Tower (1943) in the form of a theory of spatial localization, similar to an earlier theory of Bethe (1895) Based upon experimental examination of the responses to mechanical stimulation of single pain fibers of the cornea, and justifiably applied to other areas of the body surface and to other modalities, Tower employs three findings, that the neurological unit is one fiber and its ramifications, without anastomosis between fibers, that the ramifications of single fibers overlap widely, and that different regions of the area of ramification of a given fiber show differences in the properties of adaptation, excitability, refractoriness, etc If then a point stimulus is near threshold intensity, only one fiber may be activated, and even if projection to the cortex were sufficiently discrete to permit of the discrimination of one fiber's responses, the area of distribution of that fiber is too extensive to permit as precise localization as stronger stimuli are found to make possible If now the stimulus is increased, different fibers with endings near the point will be activated to characteristic patterns of impulse discharge, which depend on the region of each fiber's distribution that coincides with the stimulated point The central brain must then recognize as a sensory "point" that pattern of different responses in overlapping fibers, and not the whole area reached by branches of one or all of the fibers activated A given fiber might then be utilized in the identification of

points anywhere within its distribution, its activity contributing to different patterns depending on what other fibers were activated with it, and to what degree Following nerve block or destruction when the concentration of endings from other nerves at the margin of the denervated area is reduced, localization is less precise because fewer fibers can contribute to an adequate pattern of response The sensory spot is thus a central interpretation of a peripheral configuration of activation This local "sensory spot" configuration is presumed to consist of a central region of one fiber's distribution where this fiber is most sensitive and can respond maximally, surrounded by the terminal ramifications of other fibers whose responses will be less vigorous to a given stimulus, in a more or less heterogeneously overlapping peripheral network.

Minor refinements of this idea may be in order as more details become available concerning the distributions of nerve endings in different tissues, without rendering the basic proposal less plausible In the skin of the arm, for instance, after removal of the papillary layer of dermis containing most of the pain and touch terminals, discrete nerve twigs are found upon stimulation of the cut surface, and can be demonstrated histologically during regeneration of the pain spots (Bishop, 1944b), the center of a pain spot developing over each growing twig Accounting thus to some extent for the anatomical individuality of pain spots, their physiological individuality is still conditioned by the circumstances noted above, and in fact successive anesthesia of adjacent nerve branches with overlapping distributions reveals that some spots at least are partially blocked from each nerve, in confirmation of the histological observation (Weddell, 1941) that many sensory units are multiply innervated from branches of separate fibers This again demands that localization must depend on more than the activation of one fiber, and suggests that the anatomical arrangement into peripheral twigs, of whatever fiber source, accomplishes a local concentration of endings suitable for the activation of a pattern of stimulation that can be effectively discriminated

It is obvious here that the idea back of the term "sensory unit" has been a confused one Actually we may speak of three types of units in this connection There is first the anatomical neurone unit, consisting of one fiber and its more or less extensive ramifications, overlapping with but insulated from every other unit in the periphery Secondly, there is a structural unit consisting of the endings supplied by terminal fiber branches of one nerve twig to an overlying area of skin, the sensory spot, characterized by having a most excitable point at its center and a less sensitive periphery Third may be specified the physiological unit locus, which is the lower limit of area whose position on the body surface can be identified, or whose discreteness from adjacent units can be recognized in consciousness. The latter unit is variable, not only over different regions of the body but depending on the intensity of stimulation, sequence of stimuli and other factors The structural unit supplied by one nerve twig and recognizable as a sensory spot is perhaps the lower limit of the physiological unit The ramification of a single fiber might be considered the lower limit of a sensory spot, when such a structure is found in isolation from overlapping fibers When

such simple sensory spots are found, as for instance in the margin of a denervated area, spatial discrimination is poor, and intensity perception and affect are abnormally acute This may be another aspect of the tendency noted above for spatial discrimination and spatial summation of intensity to exclude each other, and its analysis may contribute to an understanding of the "burning pain" of the thalamic syndrome and similar states resulting from impaired innervation

The phenomena of spatial discrimination may offer a practical approach to a more general understanding of sensory processes Kellgren has suggested (1939) that pain from various parts of the body might be classified on such a basis He points out that there are three *layers* of sensation, the skin where discrimination is most precise being the first, below which, in a second layer of deep fascia, periosteum and ligaments, localization is of only fair accuracy, not equal to that of the skin From all structures below this second layer pain is diffuse, poorly localized, and liable to be referred within a segmental distribution It is referred, in fact, to those regions of the segmental distribution *in which pain is better localized*, suggesting that the segmental distribution of diffuse pain, i e , the diffusion of pain reference to other parts of the distribution than its origin, is simply a false or imperfect localization It will presumably be true that rather than three distinct layers, a gradient or transition exists from surface to viscera, and even from one area of surface to another, and that the ability to discriminate space will be a correlate both of the density of innervation and of the experience of the individual, i e , of training At least it can be said that precision of localization varies with the probability of specificity of stimulation, those parts of the body which are exposed to the most localized or definitely patterned stimulation, the hands for instance, being the ones which are capable of the finest discrimination

This concept would seem to be applicable to other senses than pain, particularly to the contact sense of touch, and especially to the various combinations of senses that give us our casual sensations What Tower's theory proposes for touch or for pain, that what the cortex recognizes as a unit locus is not a local stimulation merely but a specific pattern that is aroused by stimulation at a given point, is even more applicable to the more complex pattern involving *both* pain and touch, plus other modal varieties to be fused into a discriminating recognition of the character of the stimulating object To take an example from common experience and universally accessible, a mild itch is less accurately located than a severe one, it is more accurately located on the hand or face than on the back or thighs, and localization is extremely facilitated anywhere by light touch, particularly by successive touch say from a moving finger The tendency to manipulate or press upon poorly localized deep painful regions arises perhaps from an indefinite impulse to better localize the pain, by adding to it other more critical sensations Such combined sensations of which pain is a component are more easily bearable than is pain by itself, and the distressing character of visceral pain may be in part assigned to this inability to localize it precisely, that is, to fully evaluate it in total experience

It may be concluded in fact that only when the activation of one modality

predominates to such a degree as to exclude lesser activities from consciousness, or except where the same process of exclusion is accomplished by deliberate or attracted "attention", or when both factors are brought into play as under most experimental conditions, is one justified in treating any one modality of sensation as a discrete phenomenon Otherwise consciousness seems to pay much less heed to the concept of modalities than do the physiologists who try to differentiate between sensory processes

## SUMMARY

It appears to the writer then that the following statements are for the present tenable

1 Both sensation as a central phenomenon, and the activity of sense organs in the periphery, may be conveniently reduced to a common denominator in terms of the pattern of non-specific impulses in the fibers of peripheral nerves

2 The concept of sensory modality has become a straight-jacket from which the study of sensation may profitably be released, to the extent of relegating this vestment to the status of a loose fitting generalization

3 The pathway to the higher sensory centers is interrupted at successive levels of synaptic regions, where two processes can intervene First, individual fibers can interact to accomplish spatial summation and mutual facilitation Second, collateral activation can result in reflex or other activity characteristic of the given level

4. Each class of sense organs activates fibers within size ranges whose maxima at least are significantly characteristic, although the margins of the groups overlap Touch and light pressure show two maxima, in the beta and delta fiber ranges Pain shows two, in the delta and C ranges Warmth has one just below pain in the delta group, and cold has one just below warmth, and temperature cannot be excluded at present from a second representation in the C group The "waves" of classic nomenclature may in fact be the result of gross summation of such functional groups The double representation of certain functions in two fiber positions may mean that two aspects of these senses differ in their physiological significance, although giving rise to one sensation in consciousness when aroused undiscriminatively The utility of fiber size distribution is still a subject for speculation, and is widely so employed

5 The segregation of sensory pathways on their entrance to the cord seems to be related chiefly to reflex function at the cord level, rather than to sensation at the cortical level

6 From the point of view of utility to the organism the different reactions at different levels of a given sensory pathway are consistent and directed toward a common end. One may speak of modalities of reflex action as justifiably as of sensation Sensation is only the final response of such a pathway at the highest level, and is not necessarily its most useful or physiologically significant one

7 All the sensory pathways find expression at the reflex, thalamic and other levels The cortical projection area of a sensation is not necessarily the cortical locus of that sensation The sensory process presumably involves a considerable

amount of cortex and thalamus and possibly of lower centers, and the remainder of crude sense following injury or removal of one region should be looked upon as involving a defect in the normal sensory response, rather than as isolating the focal representation of that sense The "thalamic syndrome" may be viewed as a defective registration of pain, and similar overaction to painful stimulation may result from defects in the periphery

8 Pain is unique in the degree to which its arousal overflows into affective or emotional protest, although other senses also share this capacity, which may be accentuated or depressed in abnormal mental conditions. Below a certain intensity, whose level varies both with mental and emotional states and under anesthetics and analgesics, pain may be perceived without significant affective reaction Below the intensity of stimulation required for pain, activation of pain endings induces sensations qualitatively different from pain, as prick, itch, etc , depending also on the pattern of stimulation These non-painful "pains" fused with touch and temperature may contribute to the complex sensations of casual experience not recognized as partaking of the character of pain

9 Sensation is a function of the pattern of responses of the nerve fibers reaching the higher centers This pattern, perhaps modified at successive synaptic levels, is ultimately determined by the pattern of peripheral stimulation The different classes of sense organs are not only preferentially susceptible each to its characteristic form of energy (mechanical force, temperature, etc ) but also have properties of their own which affect the pattern of response in their nerve fibers Touch, pressure and pain for instance show different degrees of adaptation and after-discharge to a steady stimulus, and thus set up characteristic patterns of impulses in the peripheral nerves This emphasizes the fact that all parts of each sensory pathway are adapted to the useful performance of the particular type of function served

10 The precision with which sensation may be localized in the body, that is with which sensation may detect which part of the body is being stimulated, varies with the different senses, over different regions of the body, and at different depths below the surface The capacity to localize accurately may be correlated with the utility of the part in localizing objects in space, as well as with the density of innervation Sensation in a part incapable of precise localization may be referred to the region within its own segmental distribution better able to localize "Referred" pain is then simply pain in a region poorly localizable Since localization over the more sensitive areas of the body is more precise than the rather wide distribution of the endings of single fibers would account for, it is necessary to account for a localizable sensory spot as being determined by the pattern of sensory discharges set up by a point stimulus in any nerve fibers part of whose sensory endings lie within the spot Consistent with this concept, a weak stimulus activating only part of these endings to an incomplete pattern is poorly localized In casual experience where more than one "sensation" may be aroused by a given stimulus object, the whole is fused to a complex sensation in which each component serves as a cue to the interpretation of the others, the more complete pattern of activity being again the more accurately localizable

## REFERENCES


ADRIAN E D Afferent impulses in the vagus and their effect on respiration J Physiol 79: 332, 1933

Afferent discharges to the cerebral cortex from peripheral sense organs J Physiol 100: 159 1941

ADRIAN, E D AND D W BRONK The discharge of impulses in motor nerve fibers J Physiol 66: 1, 1928

ADRIAN, E D MCK CATTELL AND H HOAGLAND Sensory discharges in single cutaneous nerve fibers J Physiol 72 377, 1931

BETHE A Die Nervenendingungen in Gaumen und in Zunge des Frosches Arch f Mikr Anat 44 185 1895

BISHOP G H Responses to electrical stimulation of single sensory units of the skin J Neurophysiol 6 361 1943

The peripheral unit for pain. J Neurophysiol 7 71, 1944a

The structural identity of the pain spot in human skin J Neurophysiol 7 185, 1944b

BISHOP G H AND P HEINBECKER The afferent function of the non myelinated or C fibers Am J Physiol 114: 179 1935

BRONK, D W Fatigue in the sense organs of muscle J Physiol 67: 270 1929

The mechanism of the sensory end organ Res Publ Assn Res Nerv Ment Dis 15 60, 1934

The activity of nerve cells Cold Spring Harbor Symp on Quant Biol 4: 1 1936

BRONK, D W AND G STELLA The response to steady pressure of single end organs in the isolated carotid sinus Am J Physiol 110 708 1935

BURRILL, D Y F R GOETZEL AND A C IVY The pain threshold raising effects of amphetamine J Dent Res 23: 337, 1944

CLARK, D J HUGHES AND H S GASSER Afferent functions in the group of nerve fibers of slowest conduction velocity Am J Physiol 114: 69 1935

FREY M VON Untersuchungen ueber die Sinnesfunctionen der Menschlichen Haut Abh d Math Physical Classe der Konigl Sächs Ges der Wiss 23 175, 1896

GALAMBOS R. Inhibition of activity in single auditory nerve fibers by acoustic stimulation. J Neurophysiol 7 287, 1944

GASSER, H. S Conduction in nerves in relation to fiber types Res Publ Assn Res Nerv Ment Dis 15 35, 1935

Pain producing impulses in peripheral nerves. Res Publ Assn Res Nerv Ment Dis 23 44 1943

GASSER, H S AND J ERLANGER. The rôle played by the sizes of the constituent fibers in a nerve trunk in determining the form of its action potential wave Am J Physiol 80: 522 1927

GOLDSCHEIDER, A Zur Lehre von den specifischen Energien der Sinnesorgane Diss Berlin 1881

HARDY J D, H G WOLFF AND H GOODELL. Studies in pain, a new method for measuring pain threshold observations on the spatial summation of pain. J Clin. Investigation 19: 649 1940

Pain threshold in man Res Publ Assn Res. Nerv Ment. Dis 23 1 1943

HARTLINE, H K The response of single optic nerve fibers of the vertebrate eye to illumination of the retina. Am J Physiol 121: 400 1938

HARTLINE, H K AND C H GRAHAM Nerve impulses from single receptors in the eye J Cell Comp Physiol 1: 277, 1932

HEAD, H AND G HOLMES Sensory disturbances from cerebral lesions Brain 34: 102 1912

HEINBECKER P AND S H BARTLEY Action of ether and nembutal on the nervous system J Neurophysiol 3: 219 1940

HEINBECKER, P , G H BISHOP AND J L O'LEARY Pain and touch fibers in peripheral nerves Arch Neurol Psychiat 29 771, 1933
Analysis of sensation in terms of the nerve impulses Arch Neurol Psychiat 31 34, 1934
HEINBECKER, P , J L O'LEARY AND G H BISHOP Nature and source of fibers contrib uting to the saphenous nerve of the cat Am J Physiol 104 23, 1933
HOGG, B M Slow impulses from the cutaneous nerves of the frog J Physiol 84 250, 1935
KELLGREN, J H On the distribution of pain arising from deep somatic structures with charts of segmental pain areas Clin Sci 4 35, 1939
LEWIS, T AND E E POCHIN The double pain response of the human skin to a single stimulus Clin Sci 3 67, 1937
LORENTE DE NÓ, R Studies on the structure of the cerebral cortex 11 Continuation of the study of the ammonic system J Psych u Neur 46 113, 1934
MICHELSEN, J J Subjective disturbances of the sense of pain from lesions of the cerebral cortex Res Publ Assn Res Nerv Ment Dis 23 86, 1943
NAFE, J P Toward the quantification of psychology Psych Rev 49 1, 1942
NAFE, J P AND K S WAGONER The nature of pressure adaptation J Gen Psychol 25 323, 1941
O'LEARY, J L A structural analysis of the lateral geniculate nucleus of the cat J Comp Neurol 73 405, 1940
PENFIELD, W AND E BOLDREY Somatic motor and sensory representation in the cerebral cortex of man as studied by electrical stimulation Brain 60 389, 1937
RANSON, S W The course within the spinal cord of the non-myelinated fibers of the dorsal roots J Comp Neurol 23 259, 1913
RIVERS, W H R AND H HEAD A human experiment in nerve division Brain 31 323, 1931
SCHUMACHER, G A The influence of inflammation on the pain threshold of the skin of man Res Publ Assn Res Nerv Ment Dis 23 166, 1943
SCHUMACHER, G A AND H G WOLFF Experimental studies in headache, contrast of histamine headache with headache of migraine, etc Arch Neurol Psychiat 45 199, 1941
TALAAT, M The effect of ions on the cutaneous sensory endings of the frog J Physiol 79 500, 1933
TOWER, S Units for sensory reception in the cornea J Neurophysiol 3 486, 1940
Pain, definition and properties of the unit for sensory reception Res Publ Assn Res Nerv Ment Dis 23 16, 1943
WALKER, A E Central representation of pain Res Publ Assn Res Nerv Ment Dis 23 63, 1943
WATTERSTON, D Observations on sensation J Physiol 77 251, 1933
WEDDELL, G The pattern of cutaneous innervation in relation to cutaneous sensibility J Anat 75 346, 1941
WOLFF, H G Some observations on pain Harvey Lectures, 39, 1943–44
WOLFF, H G AND H GOODELL Relation of attitude and suggestion to perception of and reaction to pain Res Publ Assn Nerv Ment Dis 23 434, 1943
WOLFF, H G AND J D HARDY Studies on pain, observations on pain due to local cooling and on the factors involved in the "cold pressor" effect Res Publ Assn Res Nerv Ment Dis 23 123, 1943
WOLFF, H G , J D HARDY AND H GOODELL Measurement of effect on pain threshold of various substances J Clin Investigation 20 63, 1941

# THE ABSORPTION OF TRIGLYCERIDE FAT FROM THE INTESTINE

A C FRAZER

*University of Birmingham, England*

The Lipolytic Hypothesis of fat absorption has been put forward in its most complete form by Verzar and McDougall (1936) (1) On the basis of a number of experimental findings which could not be reconciled with this conception of the mechanism of fat absorption, Frazer (1938) (2) suggested the Partition Hypothesis, and subsequent work has furnished added support for this alternative view The essential points of difference between these two hypotheses are shown in table 1 It will be seen that there is general agreement on the main changes which fat may undergo during absorption Thus, the occurrence of emulsification, hydrolysis and phosphorylation, and the importance of bile salts and the adrenal cortex, are recognised by both groups of workers The differences lie in the way these changes are brought about, and in their significance and relationship to the absorptive mechanism The most recent assessment of evidence (Bloor, 1943) (3) was compiled before the full publication of the experimental work upon which the Partition Hypothesis is based Reassessment, therefore, seems desirable, and in this review the differences of opinion shown in table 1 are analysed by the critical examination of the experimental methods used, the results obtained and their interpretation in each group of problems Attention is concentrated on the mechanism of absorption of triglyceride fats The related problems of phospholipid and cholesterol absorption are not considered except in so far as they may be concerned with the absorption of triglyceride

*What changes does fat undergo in the intestinal lumen?* The two main changes which may affect fat in the intestinal lumen are emulsification and hydrolysis. The possible extent and mechanism of each of these changes can be studied either in vitro, by experiments in which the various factors concerned are investigated under controlled, but relatively static conditions, or in vivo, by the examination of material removed from the intestine Certain inferences especially with regard to the functions of these changes, can only be drawn from a more extensive survey of the experimental field.

*Emulsification* Ingested fat is finely emulsified in the upper part of the small intestine so that the particles average less than 0 5 $\mu$ in diameter It is now generally accepted that the reaction of the contents of the duodenum and jejunum is acid—about pH 6 5 (4) While more alkaline material may sometimes be found, especially in the lower ileum, it is clear that the intestinal emulsifying system must be effective at pH 6.5 Adequate criteria should be applied in experiments designed to demonstrate possible intestinal emulsifying systems The particle size and stability should be checked microscopically under dark ground illumination, and the system should be tested under conditions similar to those prevailing in the intestine The system should induce spontaneous emulsification without undue agitation and, if particulate absorption of fat is being

TABLE 1

*Main points of difference between lipolytic and partition hypotheses*

| | LIPOLYTIC HYPOTHESIS (1) (3) | PARTITION HYPOTHESIS (2) (73) (47) (31) (7) |
|---|---|---|
| 1 Changes occurring in the lumen of the intestine | | |
| (a) Emulsification | | |
| i Mechanism | Acid soap | Triple complex fatty acid/bile salt/lower glyceride (7) |
| ii Function | To promote hydrolysis | To disperse unhydrolyzed fat preparatory for absorption |
| (b) Hydrolysis | | |
| i Extent | Complete | Partial |
| ii End products | Fatty acid and glycerol | Fatty acid, di- and monoglycerides and later glycerol (8) |
| iii Function | Essential preliminary to absorption | Provides $\frac{2}{3}$ components of emulsifying system Partitions fat into fatty acid and glyceride fractions |
| 2 Passage through the outer membrane (free border) of the intestinal cell | | |
| (a) Structure of brush border | Solid pavement | Canal structure (44) |
| (b) Fatty acids | Pass through the membrane as soluble complexes with bile salts (39) | Pass through the membrane either as soluble compounds or complexes |
| (c) Glycerides | Do not pass through the membrane | Pass through the membrane as finely dispersed emulsion of negatively charged particles less than 0 5$\mu$ in diameter (7) |
| (d) Adrenal cortex | Not concerned here | Controls normal electrolyte balance which is closely related to the absorption of charged particles (27) |
| 3 Changes occurring in intestinal cell | | |
| (a) Resynthesis | Essential part of mechanism All fatty acid converted back to triglyceride | Not an essential part of the absorptive mechanism |
| (b) Phosphorylation | A stage in resynthesis of triglyceride fat (74) (75) | Not concerned with resynthesis but occurs at oil/water interface, as an essential change in interfacial structure, and probably elsewhere (10) |
| (c) Adrenal cortex | Essential for normal phosphorylation (55) | Not concerned in phosphorylation (61) |

TABLE 1—*Concluded*

| | LYPOLYTIC HYPOTHESIS (1) (3) | PARTITION HYPOTHESIS (2) (73) (47) (31) (7) |
|---|---|---|
| 4 Distribution of absorbed fatty substances in the body | | |
| (a) Fatty acid fraction | After resynthesis the triglyceride passes up lacteals and thoracic duct into systemic circulation<br>Negligible amounts pass up portal vein to liver | Mainly passes by the portal vein to the liver (2) (31) (47) |
| (b) Glyceride fraction | Not absorbed as such Resynthesized from fatty acid and glycerol as described above | To systemic blood via lacteals and thoracic duct and so to the fat depots (47) |

considered, the droplets should be smaller than 0 5 $\mu$ in diameter, and stability should be maintained for at least 3 hours.

It has been suggested that the bile salts provide an effective emulsifying system for fat in the intestine It can easily be shown that purified bile salts will only give crude emulsification with low stability not comparable with that known to occur in the intestine Other authorities have attributed emulsification in the intestine to soap (5) While soap is an excellent emulsifying agent at alkaline pH, it will not cause fine emulsification at any pH level more acid than 7.5 The suggestion that acid soaps will cause fine emulsification cannot be supported experimentally and such a system is quite ineffective in the presence of calcium. Soap is a much more effective emulsifying agent if it is formed in situ at the oil/water interface. This, of course, occurs during lipolysis. Its action is also enhanced by the presence of cholesterol due to the formation of soap/cholesterol complexes (6) None of these modifications, however, will alter the pH-conditioned character of soap emulsification. If the possible factors, such as fatty acid (soap), cholesterol, bile salts and monoglycerides, are systematically examined, singly, or in double or triple combination, only one system fulfills the criteria described above and is effective over the pH range of 6 0 to 8 5 This system is the triple combination of fatty acid/bile salt/monoglyceride (7) It can also be shown that adequate quantities of monoglyceride are formed during the first 5 hours of pancreatic lipolysis both in vitro and in vivo (8)

Material from the intestinal lumen may be examined analytically, or the interfacial structure may be studied by determining the protein flocculation pattern (9) or the effect of enzymes The emulsion is found to contain fatty acids, and tri , di and mono-glycerides, no phospholipid could be demonstrated. The flocculation pattern of the intestinal emulsion is that of simple negatively charged particles (10) which are not affected by Cl. welchii lecithinase (11) These findings would fit in with the conception that the fat emulsion particles in the intestinal lumen are stabilised with lower glyceride and fatty acid/bile salt complexes.

It may be concluded that of the various emulsifying mechanisms suggested, it is only the triple combination of fatty acid/bile salt/monoglyceride which has been shown to be effective under the conditions prevailing in the intestine The various components of this system are readily available due to lipolysis and bile secretion Phospholipid, cholesterol and other substances may, perhaps, be secondarily involved in the interfacial film, but it seems that they are not essential to the fine emulsification of fats in the intestinal lumen The functions of this emulsification may be to provide an increased interfacial area for subsequent hydrolysis, or it may be concerned with the absorption of finely dispersed fat in the unhydrolysed state The discussion of these two possibilities must be deferred until other points in the evidence have been reviewed

*Hydrolysis* "Lipase" may be one enzyme with many different actions, one or other of which may be emphasized by different combinations of activators and inhibitors, or it may consist of a group of esterases For the present it is more convenient to consider these actions as being due to different enzymes, and the term "lipase" may, with advantage, be restricted to those which mainly act on the glycerides of the long-chain fatty acids Many of the observations on the action of pancreatic lipase have been made using esters of butyric acid as the substrate While this may be convenient for enzyme studies, it is unwise to apply such experimental findings unreservedly to the problem of long-chain triglyceride hydrolysis, since separate enzymic activity toward these various substrates can be differentiated (12), (13), (14), (15), (16) and butyric acid is water-soluble in contrast to most other fatty acids

The main source of lipase in the intestinal lumen is the pancreas, although some may also be demonstrated in other intestinal juices or derived from bacteria (17) The initial flow of pancreatic juice, which is probably neurogenic, is rich in lipase, whereas the flow due to secretin has a poor enzyme content (18) Harper and Raper (1944) (19) have described another hormone, pancreozymin, which stimulates the production of amylase and trypsinogen, increase of lipase has also been demonstrated (20) It is generally agreed that ingested triglyceride is hydrolysed by lipase in the intestinal lumen The extent to which hydrolysis occurs and the nature of the cleavage products in the intestine are the main points upon which opinion differs

The percentage hydrolysis of triglyceride by pancreatic lipase in vitro is found to be influenced by the length of the experimental period, the relative amounts of oil and lipase and the degree of dispersion of the former, the presence of activators and the pH of the continuous phase, and the accumulation of cleavage products The relationship of these factors to other evidence and their possible significance in the intestinal lumen must be assessed

Observations of lipolysis may be made over any time period, but it is only the changes which occur during the first few hours that are of significance in relation to the absorption of fat from the intestine It has been frequently stated that the peak of fat absorption in the human subject is reached some 6 hours after taking a meal containing fat This is only true when excessive amounts of fat are ingested If normal quantities, such as 30 grams are taken, the

maximum systemic lipemia usually occurs in 2 to 3 hours and the blood fat levels have returned to normal within 4½ hours (21) The delay in the case of high fat diets is due to the increase in stomach emptying time In any case the significant time period for intestinal lipolysis is less than 6 hours, since the fat is not in the small intestine for any longer period

The initial stages of lipolysis may be rapid, but the rate soon declines and comes almost to a stop before 30 per cent of the contained fatty acids have been liberated. The addition of more lipase causes no increase in hydrolytic rate, but addition of more substrate may do so (16), although this will not increase the percentage hydrolysis Changing the relative proportions of oil to lipase alters the extent of hydrolysis which occurs in a given time, but the final percentage is not raised above the limiting value The quantity of fat ingested by dogs or human subjects may be increased up to 200 to 300 grams/day without any significant diminution of the percentage absorbed (22), (23), (24) A relative increase of lipase to oil may be achieved either by the passage of small amounts of oil into the intestine, by adaptation of the composition of pancreatic juice or by the administration of additional enzyme The increase in emptying time of the stomach due to excessive intake of fat might be an example of the former mechanism Chylomicrographs (25), (26) made under these conditions, however, show a shift of the lipaemic curve to the right rather than any tendency for the lipaemia to be fractionated over a longer period (27) There is evidence that only the initial flow of pancreatic juice contains much lipase and that the subsequent flow consists mainly of water and salts (18) If this is the case, no great change in the relative amounts of oil and lipase will result. The evidence on the adaptation of the enzymes of the pancreatic juice to dietary intake is conflicting and this problem requires further investigation (28), (29), (30)

The addition of potent lipase to a standard fatty meal causes suppression of the normal post-absorptive lipaemia and other changes which suggest that the percentage hydrolysis has been increased (31) This can only be explained at present on the assumption that the amount of lipase normally present is very small, or that special conditions are created which allow a high percentage to occur The limitation of hydrolysis in vitro can be readily demonstrated even if the oil is finely dispersed to a degree comparable with that found in the intestine (32) It does not seem likely therefore that fine emulsification alone can explain the occurrence of the high percentage hydrolysis required by the Lipolytic Hypothesis. Furthermore, if absorption of particulate fat occurs, the finely dispersed material will be rapidly removed from the intestinal lumen.

Willstätter and his colleagues (33) demonstrated several substances which activate lipase, of which the most important are the bile salts, albumin and calcium oleate These substances are probably non-specific activators which help to bind the water soluble lipase to its insoluble substrate Elaborate systems of activators can be built up which will stabilize lipase activity While this is necessary for the study of enzyme kinetics, the findings of such experiments are often not applicable to the systems found in the intestinal lumen. Even if these

activators are used, the percentage hydrolysis is not increased in the significant time period except under special circumstances such as those devised by Balls, Matlack and Tucker (34) There is no evidence of any complex activating mechanism necessary for lipolysis in the small intestine Hydrolysis and absorption of fat appear to be normal in the absence of any significant quantities of albumin or calcium oleate Absorption is interfered with in the absence of bile salts, but this can be well explained on other grounds, and the percentage hydrolysis of fat under these conditions is often normal (35)

The decreasing rate of hydrolysis has been attributed to the establishment of equilibrium due to the accumulation of cleavage products in the water phase, and it is claimed that in the intestine the removal of fatty acid and glycerol by absorption will allow hydrolysis to proceed rapidly to completion It is doubtful, however, whether it is justifiable to apply the Law of Mass Action in this way to a non-homogeneous system where many other factors are obviously involved in the determination of hydrolytic rate One factor which has not received sufficient attention is the relationship of the amounts of fatty acid and lipase to the available oil/water interface As hydrolysis proceeds the interface must become loaded with oil-soluble cleavage products, especially those which contain hydrophilic groups It is inevitable that an accumulation of fatty acid will tend to displace the enzyme from the oil/water interface with consequent interference with hydrolysis This effect can only be avoided if the cleavage products can be removed from the interfacial film They might be shifted either into the oil phase or into the water phase The former could be achieved in the case of fatty acid by suppression of ionization of the carboxyl groups, but this would also result in the breaking of the oil-in-water emulsion The fatty acid might be removed into the aqueous phase in three ways, as fatty acid, soap, or water-soluble complexes Only the lower fatty acids, such as butyric acid, are water-soluble, and it may be significant that tributyrin behaves differently from the long-chain triglycerides on hydrolysis both in vitro and in vivo (36), (37), (38) The removal of fatty acid as soap depends on pH, and any extensive removal of fatty acid from the oil/water interface at the intestinal pH of 6 5 cannot be demonstrated in vitro (27) In a more alkaline environment this mechanism might be more effective Fatty acids form complexes with bile salts, which are water-soluble and diffusible through artificial membranes (39) These diffusion experiments have been criticised (40), (41) but in any case the molecular relationships necessary for the formation of these complexes bear little resemblance to those prevailing in the intestinal lumen Verzar (1) pointed out that the amount of bile salt available in the intestine was not sufficient to account for the absorption of fatty acids by this mechanism, and he suggested that this objection might be overcome if the bile salts exerted their hydrotropic action at the surface of the intestinal cell This finds little theoretical or experimental support, and it provides no answer to the problem of the removal of fatty acid from the oil/water interface It may be concluded that the interface will be cleared of fatty acids if they are water-soluble, or if the pH of the environment is sufficiently alkaline,

but that under other circumstances, accumulation at the interface with consequent interference with hydrolysis is likely to occur Glycerol, if formed, will readily pass into the water phase and other possible end products such as di and mono-glycerides are alternative substrates to triglyceride and will not cause interference

It is generally stated that the end products of hydrolysis of triglyceride with pancreatic lipase are fatty acid and glycerol. If pancreatic lipolysis is carried out under standard conditions without the introduction of excessive quantities of activators or an abnormal pH level, it can be shown that free glycerol is not liberated during the first 5 hours During this period fatty acid is set free and the glyceride residue shows an increase in acetyl value from 4 to 64 If this is interpreted as being due to the formation of mono- and di-glycerides, the whole of the glycerol is readily accounted for It can also be shown in control experiments that glycerol is not destroyed during this time period, but that it does disappear over longer periods. That the end products in these experiments consist of fatty acid and mono- and di-glyceride has been further confirmed by the separation of the lower glycerides from the glyceride residue, and comparison of the compounds obtained with authentic specimens If material from the lumen of the intestine is examined, it appears to have a comparable content of monoglyceride (8) If the end products of hydrolysis during the first 5 hours are fatty acids and glycerides, and over 90 per cent of fat is absorbed during this period, it is obvious that a significant proportion of the fat must be absorbed as unhydrolysed or partially hydrolysed triglyceride.

Material obtained from the intestinal lumen may be analysed and the proportion of fatty acid determined (42) An increase in the proportion of fatty acid has been interpreted as evidence of progressive lipolysis Arguments have also been advanced in support of a high percentage hydrolysis on the basis of the fatty acid content of faecal fat, which is usually 70 to 80 per cent (43) In each of these instances it has been assumed that the only fatty material being absorbed is fatty acid If this assumption is not made and the possibility of absorption of unhydrolysed fat is considered, these observations cease to have any such significance

It is concluded that pancreatic lipase does not work in the intestine under the ideal conditions which are frequently provided in vitro The pH of the upper part of the small intestine, the short time period and the difficulty of removal of fatty acid from the oil/water interface, will all tend to limit the extent of hydrolysis. In the lower ileum, where the reaction may be more alkaline, it is possible that more extensive hydrolysis might occur and this may act as a safeguard ensuring almost complete absorption of fat Not only is the percentage hydrolysis likely to be restricted, but there is evidence that the individual triglycerides may be only partly hydrolysed to di and mono-glycerides, which, with the bile salts, provide an effective emulsifying system Fine emulsification of fat will not alone influence the eventual percentage hydrolysis, but it may allow unhydrolysed fat to be removed from the intestinal lumen by particulate absorption, the evidence for which will be discussed later If the finely dispersed fat is not removed and

the reaction of the aqueous phase becomes more alkaline or if the fat is a short-chain triglyceride, such as tributyrin, more extensive hydrolysis would be expected

*How does fat pass through the outer membrane (free-border) of the intestinal cell?* The structure of the outer membrane, the so-called brush-border, of the intestinal cell has been investigated by histological methods, but interpretation under high magnification is difficult The final conclusion on the structure of such a membrane must depend not only on its histological appearance, but also upon the results of functional permeability tests Histological examination of the membrane has been carried out by a number of observers, whose views are well summarized by Baker (1943) (44) From the study of the intestinal cells mainly of amphibia, Baker submits convincing evidence that the brush-border contains a system of fine canals, running at right angles to the surface, through which particles rather less than 0 5 $\mu$ in diameter might pass His preparations show these canals in both longitudinal and transverse section The constancy of his observations, in preparations using different methods, makes it unlikely that the canals are artefacts Wotton and Zwemer (1939) (45) have published photographs which they claim show fat passing through canals in the membrane into the intestinal cell The physical chemical forces involved in this mechanism are obscure It may be concluded that the possibility of a canalicular structure in the outer membrane of the intestinal cell must be borne in mind, and that it is unwise to consider problems of fat absorption on the assumption that the outer membrane has necessarily a pavement structure or that it resembles a lipoid film (46)

The passage of fat through the outer membrane of the intestinal cell is best studied by concurrent biochemical analysis of the residual material in the intestine, and histological examination of frozen sections The investigations should be planned on a serial basis and particular attention should be given to the length of the experimental time period and to the site from which samples are taken for section Various types of glyceride, cleavage products, or unsaponifiable material, such as paraffin, may be used The fatty materials may be administered as a finely dispersed emulsion or as crude oil The degree of dispersion of emulsions must be adequately checked before and during the experiment Many emulsions must be injected intraduodenally to avoid destruction by the acid in the stomach

Fat must pass through the membrane either as glyceride or as fatty acid It is agreed that lipolysis occurs in the intestinal lumen, and the fatty acid is likely to be absorbed, provided it can be separated from the residual unhydrolysed glyceride as a water soluble compound or complex The precise mechanism by which fatty acid passes through the membrane is not known, but it is probably the same as for other water-soluble compounds The supporters of the lipolytic hypothesis claim that all the fat absorbed from the intestine must be hydrolysed to fatty acid and glycerol before it can pass into the intestinal cell It has already been shown that hydrolysis may be restricted in the intestinal lumen and that triglycerides may be only partially broken down Further, differentiation be-

tween the absorption of triglyceride and its constituent fatty acids can be demonstrated (47) and will be discussed in more detail later For these reasons the possibility of absorption of unhydrolysed fat must be considered.

Glycerides might be absorbed either in molecular dispersion or in particulate form Glycerides might be molecularly dispersed in water in the form of phosphorylated compounds The available evidence suggests that phospholipids are broken down in the intestinal lumen, and that synthesis of these compounds occurs after the passage of the fatty components into the intestinal cells No hydrotropes have been demonstrated which form an association with glycerides, so that molecular dispersion in complex form does not seem possible It must be concluded on present evidence that glycerides do not pass through the intestinal membrane in a state of molecular dispersion.

Although it has been claimed that large globules of fat pass through the intestinal membrane (45), it is difficult to accept this mechanism on histological evidence alone in the absence of any adequate physical chemical explanation If the possibility of finely dispersed fat particles passing through the membrane is to be considered, it must be demonstrated that an effective emulsifying system exists in the intestinal lumen, that the membrane structure is suitable and that finely dispersed unsaponifiable material can be absorbed The physical chemical forces required for the passage of the particles into the cell must be defined, and the relationship of this mechanism to the selective absorption of other foodstuffs determined.

The existence of an effective emulsifying system in the intestine, which will reduce the particle diameter to less than 0 5 $\mu$, has already been discussed The canalicular structure of the outer membrane of the intestinal cell described by Baker would seem to be suitable for absorption of particles of this size The absorption of unsaponifiable material, such as paraffin, has been the subject of much controversy Channon and his co-workers (48), (49) have demonstrated the absorption of paraffins, but other workers have denied that absorption occurs (50), (51), (52) Unfortunately these observations were made without adequate control of the state of division of the paraffin Statements on the absorption of emulsified material (53) are often misleading since the milky appearance of oil in water emulsions, especially if they are made with pre-formed soap solution cannot be regarded as a satisfactory criterion of fine emulsification The size of the particles in these experiments must be determined with reasonable accuracy by microscopic methods It is generally agreed that absorption of paraffin is minimal if it is administered orally either unemulsified or as a coarse emulsion If a paraffin emulsion with an average particle diameter of less than 0 5 $\mu$ is injected intraduodenally, it is absorbed in amounts comparable with a similar olive oil emulsion The absorption of these paraffin emulsions has been demonstrated by residual analyses and histological studies, and has been a constant finding in large groups of rats (7) It has been concluded in the past that paraffin was not absorbed because it was not hydrolysed Although the reasons for reaching this conclusion were probably false, it is possible that the conclusion itself was correct for hydrolysis provides two essential components of the

intestinal emulsifying system Paraffin is thus only absorbed from the intestine if it is finely dispersed with suitable emulsifying agents before administration, and dispersion is maintained in the alimentary tract

The fat particles in the intestinal lumen can be shown to carry a negative charge Teorell (54) demonstrated the relationship of electrolyte diffusion to the passage of negatively charged particles through membranes It is not impossible that the outer membrane of the intestinal cell may be charged due to difference in the diffusion rates of absorbed ions and that this may have an important bearing on the absorption of finely dispersed fat particles The relationship of such a system to the selective absorption of other substances than fat requires investigation

If electrolyte absorption is closely associated with the passage of fat particles through the outer membrane, it might be expected that double adrenalectomy, with the consequent electrolyte disturbance, might affect fat absorption Verzar and Laszt (1935) (55) claimed that fat absorption in rats was reduced to less than 10 per cent of normal following double adrenalectomy They considered that this depression of fat absorption was due to interference with phosphorylation A number of workers have confirmed that fat absorption is depressed after adrenalectomy Normal absorption was re-established by the administration of corticosterone, and it was also improved by adequate sodium therapy (56), (57), (58), (59) These and other observations (60), (61) do not substantiate the view that the adrenal cortex is concerned with phosphorylation processes in the intestinal cell In a study of the differential absorption of triglycerides and fatty acid in adrenalectomised rats (27) we found that triglyceride absorption was depressed to 40 per cent, but that fatty acid absorption was practically normal quantitatively We confirmed Verzar's observation that fatty acid absorption in adrenalectomised animals gave rise to toxic symptoms The absorption of triglyceride was improved in salt-treated animals The time of onset of defective fat absorption coincided with the development of electrolyte imbalance Bavetta and Deuel (1942) (62) have described normal absorption of tributyrin and butyric esters after adrenalectomy, and they suggest that the adrenal glands play a rôle in the absorption of the long-chain fatty acids It has already been suggested that tributyrin is likely to be absorbed more as fatty acid than unhydrolysed fat, whereas long-chain glycerides will tend to pass through mainly in particulate form If adrenalectomy interferes with the absorption of fat particles but not fatty acid, the observations of Bavetta and Deuel are readily explained

It may be concluded from these various observations that fat can be absorbed as fatty acid provided that the cleavage products can be removed into the aqueous phase from the oil/water interface This appears to be more easily accomplished in the case of butyric acid than the long-chain fatty acids It is suggested that the possibility of particulate absorption of unhydrolysed fat must be considered, especially in relation to the absorption of long-chain fats The absorption of fat particles may be dependent upon normal electrolyte metabolism, and this might account for the importance of the adrenal cortex in the absorption of long-chain compounds

These two mechanisms of absorption can be accepted on the evidence available without necessarily assuming that they both play a part in normal fat absorption, although it seems probable that this is the case The relative proportions of fat normally absorbed in the hydrolysed or unhydrolysed state have not yet been determined, but it seems likely that the ratio will vary according to the type of fat ingested, the conditions in the intestine, the normal functioning of the intestinal cells and other metabolic activities The effect of added lipase (31), (47) and the differentiation of triglyceride and fatty acid absorption suggests that, under normal circumstances, most of the fat in the chyle is derived from material absorbed in particulate form Munk and Rosenstein (63), in their classical experiments, recovered only 60 per cent of ingested fat from a lymphatic fistula, in adrenalectomised animals interference with particulate absorption causes a depression to 40 per cent of normal, and lipolysis in the significant time period tends to be restricted to about 30 per cent under the conditions found in the intestine Bearing in mind that an upset of the normal mechanism of particulate absorption may lead to an increase in the percentage hydrolysis, it seems likely that, so far as long-chain triglycerides are concerned, at least 60 per cent of the fat absorbed may pass through the outer membrane of the intestinal cell as finely dispersed particles of unhydrolysed fat

*What changes does fat undergo in the intestinal cell?* Evidence of changes in the composition of fatty material in the intestinal cell may be obtained directly by histo-chemical studies or indirectly by comparison of the substances entering and leaving the cell This evidence may be correlated with in vitro experiments which may indicate both the nature and possible function of the changes observed The interpretation of histo-chemical methods is difficult, not only for technical reasons, but also because they give a static picture of intracellular changes The available methods for the differentiation of triglycerides from fatty acids are unreliable (64) The examination of substances immediately before entering or after leaving the intestinal cell is not possible, but an approximate assessment is made by the study of the contents of the intestinal lumen and the blood or lymph leaving the intestine It is obviously an advantage if the material is collected as soon as possible after leaving the intestinal cell to minimize the danger of admixture with entirely irrelevant substances Thus, the technique of collecting intestinal lymph (65) is preferable to thoracic duct cannulation. Whatever methods are used, however, considerable caution must be exercised in their interpretation

The two main changes which are thought to occur in the intestinal cell are resynthesis of triglyceride and phosphorylation There is no doubt that most of the fat in the chyle is triglyceride If complete hydrolysis of fat occurs before absorption, then resynthesis must take place If, however, a large proportion of the fat is absorbed in the unhydrolysed state, then resynthesis is not necessarily important Various experiments have been made which purport to show triglycerides in the chyle derived from monoglycerides, fatty acids or other esters (66), (67), (68) In none of these experiments can the synthesis of triglycerides in the lumen of the intestine or the possibility of the triglycerides being derived

from some other source than immediately absorbed material, be excluded That glyceride synthesis from fatty acids and glycerol may occur in the presence of lipase can be readily demonstrated in vitro (69), (70), (71), and it seems reasonable to suppose that given suitable conditions similar changes can occur in vivo Whatever may be the true interpretation of the experiments so far devised to demonstrate resynthesis, they do no more than indicate that synthesis can occur under certain conditions They do not show the extent of triglyceride resynthesis during normal fat absorption Verzar has advanced the histological evidence of Jeker (72) as support for resynthesis He demonstrated what appeared to be fatty acid in the intestinal cell 30 minutes after ingestion, but after 6 hours the cells were seen to be full of neutral fat Quite apart from the validity of the histo-chemical methods employed, these experiments cannot be accepted as evidence of resynthesis, for identical results could obviously be obtained by early fatty acid absorption and later absorption of unhydrolysed fat (73)

Phosphorylation has been regarded as an important intermediate step in triglyceride resynthesis (74), (75), (1) Whether resynthesis occurs or not, the conception that phosphorylation is a stage in this process is open to question The reversible nature of lipolysis has been amply demonstrated in vitro, and the formation of triglyceride from cleavage products in the presence of lipase does not appear to require intermediate phosphorylation The available evidence suggests that phospholipid in the intestinal cell should be more properly regarded as an end product than an intermediate compound in resynthesis

Although the relationship between phosphorylation and triglyceride resynthesis is doubted, there is good evidence that phospholipids are formed in the intestinal cells This conclusion is based on histo-chemical studies (76) and on experiments in which labelled phosphorus or fatty acids were traced from the intestinal lumen through the intestinal cell into phospholipids leaving the intestine (77), (78), (79), (80), (81)

Verzar supported this conception of the importance of phosphorylation by experiments which showed that moniodo-acetic acid and phloridzin (82) caused interference with absorption of fat The doses used were excessively large, and can be shown to cause extensive destruction of the intestinal mucosa (83) It seems unreasonable to assume, therefore, that the interference with fat absorption in these experiments can be attributed to faulty phosphorylation Even if phospholipids are not concerned with resynthesis, there may be many other important reasons for phospholipid formation Phospholipid is a convenient transportable form of fat and it may be more suitable than triglyceride for many metabolic reactions It can also be demonstrated that phospholipid is an essential component of the stabilising film of fat particles circulating in the blood stream, and that the maintenance of the phospholipid content of the interfacial film is necessary to their continued dispersion Negatively charged fat particles flocculate in the presence of plasma proteins and 1 per cent sodium chloride at a pH of 7 5 (9) This flocculation does not occur if the particles are stabilised with phospholipid instead of soap If these two types of emulsion are treated with Cl welchii lecithinase, the soap-stabilised emulsion is unaffected while the phos-

pholipid-stabilised emulsion flocculates. If the fat particles in the intestinal lumen are examined they are found to behave as simple negatively-charged particles, but the chylomicrons on the other hand behave similarly to a phospholipid *stabilised emulsion*. *It is tentatively suggested that phosphoipid is added to* the fat particles in the intestinal cell, and it may be formed in situ at the oil/water interface. It is not impossible that monoglyceride might provide the basis for this phospholipid formation (10). Alternatively, phospholipid may be preformed in the intestinal cell and spread as a film on the surface of the oil globule.

The relationship of the adrenal cortical hormones to phosphorylation (55) has *not been substantiated by other workers either in connection with fat* (61), carbohydrate (84), (85), or vitamin (86) absorption. It is difficult, therefore, to accept this conception of the action of the adrenal gland, and a possible alternative explanation of its relationship to fat absorption, due to the associated electrolyte disturbance, has already been discussed. It may be concluded that the question of the significance of resynthesis in the intestinal cell depends upon the form in which fat is absorbed. If a large proportion of fat enters the cell as triglyceride, resynthesis ceases to be of such vital importance. The relationship of phosphorylation to resynthesis or adrenal cortical hormone is open to question. It may, however, represent an essential change in the interfacial structure of fat particles needed to maintain normal stability and dispersion in the blood stream. It is likely that phospholipid formation has other important functions not yet clearly defined.

*What is the distribution of absorbed fatty material?* This problem can only be studied by in vivo experiments. The fatty material may be labelled by staining, by the introduction of recognisable chemical groups, or the incorporation of radio-active elements. In addition to determining the immediate destination of the fatty material, the pathways along which it passes from the intestine may be studied. The two main routes are the lacteal lymphatic channel through which the chyle is passed into the systemic circulation, and the capillary network which leads via the portal vein to the liver. While fat, travelling in the systemic blood, need not necessarily be removed into the fat depots, or that in the portal blood into the liver, it seems probable that systemic lipaemia will tend to be associated with increased deposition in the depots and portal lipaemia with a rise in liver fat, provided that excessive quantities of fatty materials are not employed. In the study of the pathways of absorption repeated chemical analyses may be used, but the number of observations made is frequently restricted. Chylomicrographs, for which much less blood is required, give a comparative serial picture of blood fat changes which is often more valuable than occasional chemical analysis in the study of fat absorption (25), (21), (26), (87).

According to the Lipolytic Hypothesis fat is completely hydrolysed before absorption and all the fatty acid is reconverted into triglyceride in the intestinal cell and passes by the lacteal lymphatic route into the systemic blood. Under these circumstances it would be expected that a reasonable similarity would exist between the phenomena observed during absorption after the ingestion of triglyceride or the administration of equivalent amounts of its constituent fatty acids

glycerol This was not so, and constant differences could be demonstrated
veen groups of animals fed on olive oil and those receiving oleic acid and glyc-
In the former, the intestinal cells were filled with fat particles, the lacteals
a milky appearance, there was the characteristic post-absorptive systemic
emia, and Sudanised oil could be traced to the fat depots, the portal blood fat
not appreciably changed and only slight deposition of Sudanised material
d be seen in the liver In the animals receiving fatty acid, the intestinal cells
a granular appearance, there was no milkiness of the lacteals, there was no
-absorptive systemic lipaemia and no deposition of Sudanised material in the
depots, the portal blood, however, showed an increase of fatty material and
nsive deposition of Sudanised fat could be seen in the liver (47) In accord
i these observations it was also shown that the addition of extra lipase to
lyceride accentuated the fatty acid component of the absorption picture, and
; inhibition of lipolysis with sodium hexadecyl sulphate produced opposite
ts In the human subject the addition of lipase caused a marked reduction
ie post-absorptive systemic lipaemia (31)
hemical analysis of the portal blood is of limited value in determining the use
is pathway, since small changes may be significant and the relationship to
type of fat administered, the degree of hydrolysis and the time of sampling,
profoundly influence the interpretation of the results In spite of these
culties, however, evidence has been put forward which indicates that fat may
bsorbed via the portal vein Numerous observers (88), (89), (90), (91), (92)
e demonstrated an increase of fat in the portal blood during absorption, but
rary evidence is presented by Zucker (1920) (93), and by other workers (94),
Raper (1913) (96) suggested that the lower fatty acids might be absorbed
a different mechanism from long-chain compounds, which is of particular
rest in the light of more recent work (36), (37), (38), (62) and the points
iously discussed in this paper, which indicate the probability that the short
n fatty acids will be more easily detached from the oil/water interface
se various observations, and the apparent disappearance of 40 per cent of fat
Munk's experiment, the demonstration of 70 per cent absorption of fat in some
s of idiopathic steatorrhoea without any appreciable systemic lipaemia, the
that tributyrin is absorbed but cannot be demonstrated in the chyle (37) or
ie fat depots (36) all indicate the presence of an alternative pathway which is
y to be the portal vein It is concluded, therefore, that fat may pass by the
al vein and that this pathway is used when fatty acid is absorbed During
yceride absorption, it is the short-chain fatty acids which are most likely to
oncerned in this mechanism The observations of Winter and Crandall (94)
e been interpreted as being contrary to the Partition Hypothesis They
ved that there was no significant increase of fatty material in the portal blood
ogs during the absorption of olive oil from the intestine These findings are
pletely in accord with the Partition Hypothesis and with the observations
e in rats in which minimal changes were found in the portal blood and liver
r administration of olive oil in contrast to the marked increase of fatty ma-
il which occurred after feeding oleic acid (47)

Extensive information on the composition of the fat depots is now available (97) It is clear that depot fat may be derived partly from food fat and partly from other sources. The resemblance between the fat in the depots and the food, especially on a high fat diet, may be explained by either hypothesis of fat absorption The apparent selection of certain fatty acids for deposition in the depots is a complex problem It is possible that further studies of lipolysis, the behaviour of different fatty acids at the oil/water interface and the factors concerned in fat synthesis, may throw some light on this mechanism. The occurrence of dehydrogenases in the intestinal lumen has been the subject of conflicting reports (98), (99), (100), (101), (102), (103), and for this reason has been excluded from this discussion. It is clear, however, that this might profoundly influence the hydrolysis, absorption and distribution of fatty material.

From the evidence submitted in this review, it is suggested that the Lipolytic Hypothesis of fat absorption, in its present form, fails to explain an increasing number of observations in this field, and many of the assumptions upon which it is based are in need of reconsideration. In particular, this conception of the fat absorption mechanism maintains that fat is completely hydrolysed in the intestinal lumen, that the intestinal cell has an outer pavement membrane, that paraffins are not absorbed, that the adrenal glands control phosphorylation of fat in the intestinal cell and that no significant amount of fatty material passes up the portal vein during absorption None of these points is satisfactorily supported by the available evidence In addition, the Lipolytic Hypothesis provides no adequate explanation of the fine emulsification of fat in the intestinal lumen, the differentiation between neutral fat and fatty acid absorption, the effect of added or inhibited lipolysis, the difference between the absorption of tributyrin and long-chain triglycerides, and the improvement in fat absorption when adrenalectomised animals are adequately salt-treated. The Partition Theory has been put forward as an alternative working hypothesis upon which further investigation of the many outstanding problems of fat absorption might be based

## REFERENCES


(1) Verzar, F and E J McDougall. Absorption from the intestine Longmans Green & Co , London 1936
(2) Frazer A C Analyst 63 308 1938
(3) Bloor W R Biochemistry of fatty acids Reinhold Pub Corp New York 1943
(4) Robinson C S J Biol Chem 108 403 1935
(5) Mellanby J J Physiol 64 5P, 1927
(6) Schulman, J H and E G Cockbain Trans Faraday Soc 36 651 1940
(7) Frazer A C J H Schulman and H C Stewart J Physiol 103 306 1944
(8) Frazer A C and H G Sammons Biochem J 39 122 1945
(9) Elkes J J A C Frazer J H Schulman and H C Stewart Proc Roy Soc A 154 102 1945
(10) Elkes J J and A C Frazer J Physiol 102 24P 1943
(11) Macfarlane M G and B C J G Knight Biochem J 35 885 1941
(12) Willstätter R and F Memmen Hoppe-Seyler s Ztschr 138 216 1924
(13) Loevenhart A S J Biol Chem 2 397 415 427 1907
(14) Cherry, I S and L A Crandall. Am J Physiol 100 265 1932

(15) Wolvekamp, H P and K Z Griffioen Ztschr phys Chem **223** 36, 1934
(16) Sobotka, H and D Glick J Biol Chem **105** 199, 1934
(17) Michaelis, L and Y Nakahara Ztschr Immun Forsch **36** 449, 1923
(18) Mellanby, J J Physiol **60** 85, 1925
(19) Harper, A A and H S Raper J Physiol **102** 115, 1943
(20) Greengard, H , M I Grossman, J R Woolley and A C Ivy Science **99.** 350, 1944
(21) Frazer, A C and H C Stewart J Physiol **90** 18, 1937
(22) Rubner, M Ztschr Biol **15** 115, 1879
(23) Arnschink, L Ztschr Biol 434, 1890
(24) Rekers, P E , J C Abels and C P Rhoads J Clin Investigation **22** 243, 1943
(25) Fish, P A and S Gage Am J Anat **34** 1, 1924
(26) Frazer, A C and H C Stewart J Physiol **95** 21P, 23P, 1939
(27) Frazer, A C Unpublished
(28) Bompiani, R Arch ital Biol **58** 111, 1913
(29) Comfort, M W and A E Osterberg Am J Digest Dis **8** 337, 1941
(30) Grossman, M I , H Greengard and A C Ivy Am J Physiol **138** 676, 1943, **141** 38, 1944
(31) Frazer, A C J Physiol **102** 329, 1943
(32) Frazer, A C and V G Walsh J Physiol **78** 467, 1933
(33) Willstätter, R , E Waldschmidt-Leitz and F Memmen Hoppe-Seyler's Ztschr **125** 43, 1922
(34) Balls, A K , M B Matlack and I W Tucker J Biol Chem **122** 125, 1937
(35) Hutchinson, H S and G B Fleming Glasgow Med J **94** 65, 1920
(36) Eckstein, H C J Biol Chem **81** 613, 1929
(37) Hughes, R H and E J Wimmer J Biol Chem **108** 141, 1935
(38) Davis, R E J Biol Chem **88** 67, 1930
(39) Verzar, F and A Kuthy Biochem Ztschr **205** 369, **210** 265, 281, 1929, **230** 451, 1931
(40) Breusch, F L Biochem Ztschr **293** 880, 1937
(41) Vonk, H F , C Engel and C Engel Biochem Ztschr **295** 171, 1938
(42) Levites, S Hoppe Seyler's Ztschr **49** 273, 1906
(43) Fowweather, F S Brit J exper Path **7** 15, 1926
(44) Baker, J R Quart J micr Sci **84** 73, 1942
(45) Wotton, R M and R L Zwemer Anat Rec **75** 493, 1939
(46) Davson, H and J F Danielli The permeability of natural membranes The University Press, Cambridge
(47) Frazer, A C J Physiol **102** 306, 1943
(48) Channon, H J and G A Collinson Biochem J **22** 391, 1928, **23** 676, 1929
(49) Channon, H J and J Devine Biochem J **28** 467, 1934
(50) Henriques, V and C Hansen Zentralbl Physiol **14** 313, 1900
(51) Mellanby, J J Physiol **64** 33P, 1927
(52) Bloor, W R J Biol Chem **15** 105, 1913
(53) Verzar, F Ergebn Physiol **32** 447, 1931
(54) Teorell, T Trans Faraday Soc **33** 940, 1937
(55) Verzar, F and L Laszt Biochem Ztschr **276** 11, 396, 1935
(56) Barnes, R H , E S Miller and O Burr J Biol Chem **140** 241, 1941
(57) Clark, W G and A N Wick Proc Soc exper Biol N Y **42** 336, 1939
(58) Bavetta, L A , L Hallman, H J Deuel, Jr and P O Greeley Am J Physiol **134** 619, 1941
(59) Clark, W G and E M MacKay Am J Physiol **137** 104, 1942
(60) Ochoa, S and R J Rossiter J Physiol **97** 1P, 1940
(61) Stillman, N , C Entenman, E Anderson and I L Chaikoff Endocrinology **31** 481, 1942

(62) Bavetta, L A and H J Deuel, Jr Am J Physiol 136 712, 1942
(63) Munk, I and A Rosenstein Virchow's Arch 123: 230, 484, 1891
(64) Kaufmann, C and E Lehman Virchow's Arch. 270 360, 1928
(65) Wilbrandt, W and H Süllmann Biochem Ztschr 270: 63, 1934
(66) Franck, O Ztschr Biol 36 568, 1898
(67) Argyris, A and O Franck Ztschr Biol 59 143, 1912
(68) Müller J and H Murschhauser Biochem Ztschr 78 63 1917
(69) Pottevin, H C R Acad Sci Paris 136 767 1903
(70) Artom, C and L Reale Bull Soc Chim biol Paris 18: 959, 1936
(71) Armstrong, H E and H W Gosney Proc Roy Soc B 88 176, 1914
(72) Jeker, L Pflüger's Arch 237 1 1936
(73) Frazer, A C Physiol Rev 20 561, 1940
(74) Sinclair, R G J Biol Chem 82 117 1929
(75) Sinclair R. G Oil and Soap 15 70 1938
(76) Lison L Histochemie Animale Gauthier Villars, Paris 1936
(77) Sinclair, R. G J Biol Chem 115 211 1936
(78) Artom C and G Perretti Arch int Physiol 42: 61, 1935
(79) Perlman, I, S Ruben and I L Chaikoff J Biol Chem 122 169, 1938
(80) Hahn, L and G Hevesy C R Lab Carlsberg 22 188 1938
(81) Hevesy G Enzymologia 5 188, 1938
(82) Verzar, F and L Laszt Biochem Ztschr 270 35 1934
(83) Wood H O Proc Soc exper Biol April 1944
(84) Deuel H J Jr, L F Hallman S Murray and L T Samuels J Biol Chem 119 607 1937
(85) Althausen, T L E M Anderson and M Stockholm Proc Soc exper Biol N Y 40: 342, 1939
(86) Clark W G Endocrinology 28 545 1941
(87) Elkes J J, A C Frazer and H C Stewart J Physiol 95 68 1939
(88) Errico G Arch Fisiol 4 513, 1907
(89) Roger, H and L Binet C R. Soc Biol Paris 88: 79 1922
(90) Sicard J A, R Fabre and G Forestier Bull Soc Chim biol Paris 5: 413 1923
(91) Cantoni, O Boll Soc ital Biol sper 3 1278, 1928
(92) Nedwedski, S Pflüger s Arch 214: 337 1926
(93) Zucker, T F Proc Soc exper Biol N Y 17: 89 1920
(94) Winter I C and L A Crandall. J Biol Chem 140 97 1941
(95) Little J M and C S Robinson Am J Physiol 134: 773 1941
(96) Rafer, H S J Biol Chem 14: 117, 1913
(97) Hilditch T P The chemistry of natural fats Chapman & Hall Ltd London, 1940
(98) Long J H and F Fenger J Am Chem Soc 39 1278 1917
(99) Tangl H and N Berend Biochem Ztschr 230 234 1930
(100) Berend, N Biochem Ztschr 260 490 1933
(101) Page H, V Parternack and L Burt Biochem Ztschr 223 445, 1930
(102) Bodansky, M Proc Soc exper Biol N Y 28: 630 1931

# THE USE OF ISOTOPICALLY MARKED CARBON IN THE STUDY OF INTERMEDIARY METABOLISM

JOHN M BUCHANAN AND A BAIRD HASTINGS

*The Department of Physiological Chemistry, University of Pennsylvania, and The Department of Biological Chemistry, Harvard University*

The contribution of physics to the analytical armamentarium of chemistry in the form of isotopes has greatly extended the vision of biochemists into the understanding of the metabolism of both organic and inorganic substances in biological systems The present paper will review those experiments on the metabolism of organic compounds and of $CO_2$ in which isotopes have been employed to trace the biological adventures of carbon atoms

Two isotopes of carbon have been available for these studies, the stable isotope, $C^{13}$, often called heavy carbon, and the radioactive isotope, $C^{11}$ In many instances, carbon atoms have been tagged with stably bound deuterium and these isotopically marked organic compounds have been successfully used in the study of biological reactions

Although the use of these three types of isotopic compounds has resulted in many new and interesting facts of metabolism, they have served an even greater function by enabling biochemists to integrate and reinterpret information obtained by conventional analytical methods It is, therefore, the intent of this review to show how carbon isotopes have been of aid in developing certain branches of knowledge rather than to divide the subject matter into experiments carried out with the several isotopes In this way, it is believed that a clearer appreciation can be gained of what has been accomplished as well as what problems need next be attacked

No attempt has been made to cover thoroughly the field of bacterial metabolism since it has previously been reviewed by Werkman and Wood (145) Certain recent publications on this subject along with other older material which is pertinent to the discussion of mammalian metabolism will be included, however

The rôle that isotopic carbon has played in elucidating certain reactions of mammalian metabolism will be discussed in the light of Schoenheimer's theory of the dynamic state of the body constituents (115) Since carbon isotopes have been used particularly in the study of carbohydrate and fat reactions, special emphasis will be placed on the metabolism of these compounds

METABOLISM IN THE LOWER FORMS The detailed investigation with isotopic carbon of the utilization of $CO_2$ by bacterial and mammalian cells has removed one of the oldest distinctions between plant and animal life Although the extent of $CO_2$ assimilation and subsequent history of the carbon atoms in these two forms of life vary greatly, the occurrence of this remnant of a photosynthetic process in respiratory reactions has required the adoption of an altered point of view toward the significance of $CO_2$ in metabolism

A *Metabolism of Carbon Dioxide in Plants* The use of isotopic radioactive

carbon ($C^{11}$) as a tool of biochemical research was first introduced by Ruben, Hassid and Kamen (110) In the course of their studies on photosynthesis, Ruben and co-workers (113) isolated from plants (*Chlorella pyrenoidosa*) exposed to an atmosphere of radioactive $CO_2$, a radioactive fraction containing carboxyl and alcoholic hydroxyl groups Plants which were allowed to grow in the presence of light produced a photosynthetic intermediate which contained only a small fraction of the total $C^{11}$ assimilated in the carboxyl groups, most of the activity resided in non-carboxyl carbon atoms of the molecule In contrast, when plants were exposed to $C^{11}O_2$ in the absence of light, a fraction was isolated which contained most of the radioactivity in the carboxyl groups This finding indicates that $CO_2$ fixation occurs during the dark reaction but that the light reaction is responsible for the reduction of the carboxyl groups formed during the dark reaction It should be noted, however, that although the use of isotopic carbon provides the most direct evidence for $CO_2$ fixation in the dark reaction, previous experiments by workers in the field of photosynthesis had led them to a similar conclusion This is thoroughly reviewed by Franck and Gaffron (54)

Data obtained by ultracentrifuge and diffusion observations (112, 114) on the $C^{11}$-containing intermediate have demonstrated that this substance is an ionized molecule with a molecular weight of approximately 1,000 to 1,500 During the period of photosynthesis, there is an increase in molecular weight De spite the high sensitivity of the radioactive indicator method, the first molecules in which radioactivity was detected were of high molecular weight No trace of the presence of molecules of small molecular weight such as formic acid could be detected.

Some of the results obtained by the Berkeley workers are not readily interpretable (112) on the basis of the classical theories of Willstätter and Stoll which are founded upon two main assumptions—namely, 1, the formation of an addition compound between $CO_2$ and chlorophyll, and 2, the subsequent photochemical reduction to formaldehyde

From studies using $C^{11}$, Ruben, Kamen and co-workers support a theory of photosynthesis involving the following steps

1 The first step in photosynthesis is the addition of $CO_2$ to a large molecule of unknown composition with the formation of a carboxyl group This reaction is a thermal (non photochemical) process which is probably enzymically catalyzed. The uptake of carbon dioxide in this dark reaction is initially rapid and gradually decreases, suggesting that the reaction may be reversible Chlorophyll is considered not to be concerned with this reaction, since no relationship was found between the concentration of chlorophyll in the leaves and the amount of $CO_2$ assimilation during the dark reaction This first reaction is represented by the following equation

$$RH + CO_2 \rightleftharpoons RCOOH \qquad (1)$$

2 The second, irreversible, endothermic reaction of photosynthesis is considered to be catalyzed by chlorophyll It is represented by the equation

$$RCOOH + H_2O \xrightarrow{\text{chlorophyll}} RCH_2OH + O_2 \qquad (2)$$

Although the work of Ruben and Kamen has demonstrated that a reversible carboxylation reaction is an initial step of photosynthesis, it has been difficult to formulate the details of the reactions involved The nature of the substance "R" with which $CO_2$ combines is unknown Ruben and Kamen have indirect evidence, however, suggesting that RH may be an aldehyde A more serious obstacle, however, has been the apparent irreversibility of known decarboxylation reactions, the reversibility of which could provide a mechanism for the lengthening of a carbon chain ultimately to a hexose unit This difficulty has been partially overcome by the demonstration by Lipmann and Tuttle (83) and by Utter, Lipmann and Werkman (135) that the phosphoroclastic reaction may be reversed

$$CH_3COCOOH + H_3PO_4 \leftrightharpoons CH_3COOPO_3H_2 + HCOOH \qquad (3)$$

On the basis of these investigations, Lipmann and Tuttle (83) have proposed a theory similar in many respects to an earlier one of Ruben (109), whereby carbon chains are progressively lengthened by a series of alternating phosphorylation and photoreduction reactions

$$R\ COOH + ad \sim PO_3^{-1} \rightarrow R\ CO\ OPO_3^{=} + ad\ H \qquad (4)$$

$$R\ COOPO_3^{=} + CO_2 + H_2 \| O \rightarrow R\ CO\ COOH + HO\ PO_3^{=} + \frac{O_2}{2} \qquad (5)$$

$$R\ CO\ COOH + H_2 \| O \rightarrow R\ CHOH\ COOH + \frac{O_2}{2} \qquad (6)$$

Further phosphorylation of the carboxyl group of the compound R CHOH COOH, reductive carboxylation and hydrogenation to hydroxy acid would result in the formation of the next higher homologue, R CHOH CHOH COOH

The reducing reagent in these reactions is photolytically formed hydrogen In addition to its reductive functions, the reoxidation of one out of seven moles of photolytic hydrogen is presumed to be the source of energy required to supply the essential energy-rich phosphate bonds

For more extensive information on photosynthetic reactions and the rôle which isotopic compounds have played, the reader is referred to reviews by Franck and Gaffron (54, 55), and by van Niel (97, 98)

In an extension of their work with plants, Ruben and Kamen (111) found that fresh yeast cells suspended in water also assimilate $CO_2$ In their experiments, approximately one $CO_2$ molecule was reduced for every fifty molecules produced in respiration Though most of the radioactivity was present in the precipitate formed by the addition of barium ions, the activity did not reside in the carboxyl groups of the barium precipitable material

B *Metabolism of Carbon Dioxide in Microorganisms* Although certain forms of autotrophic bacteria may utilize $CO_2$ as the sole source of carbon in the syn-

[1] a d $\sim PO_3^{=}$ refers to a high energy phosphate group in adenosine triphosphate or adenosine diphosphate ad H refers to adenylic acid $H_2 \| O$ represents the photolytic splitting of water into hydrogen and oxygen

theas of complex organic structures, it has also been shown that heterotrophic bacteria can likewise assimilate $CO_2$

1 *Formation of organic compounds by reduction of $CO_2$* One of the simplest reactions of $CO_2$ in bacterial metabolism is its reduction to formic acid by $H_2$ in the presence of the enzyme hydrogenlyase (155)

In more complex systems, $CO_2$ is reduced to a one-carbon organic compound with the simultaneous oxidation of other metabolites (1) Thus, it has been shown by isotopic studies that *Methano-bacterium omelianski* is capable of oxidizing methanol or ethanol to their corresponding fatty acids with the simultaneous reduction of $CO_2$ to methane In a similar manner, *Methanosarcina methanica* can oxidize fatty acids, derived from methanol or other alcohols, to $CO_2$ with the concurrent reduction of $CO_2$ to methane

2 *Carbon to carbon addition by $C_3$-$C_1$ union (Wood Werkman reaction)* The first indication that heterotrophic organisms can utilize $CO_2$ with the formation of carbon to-carbon bonds came from the classical work of Wood and Werkman (148) who, using chemical methods only, were able to demonstrate an overall uptake of $CO_2$ by propionic acid bacteria during the fermentation of glycerol to succinic, propionic and acetic acids By refining the methods of analysis, they were able to demonstrate that one molecule of $CO_2$ disappeared for every mole by succinic acid formed (149)

On the basis of these results, these investigators postulated that a one-carbon compound ($CO_2$) reacted with a three-carbon compound by $C_3$ to $C_1$ union to form the four-carbon dicarboxylic acid, succinic acid Pyruvic acid was suggested as the intermediate with which $CO_2$ reacted

a *Studies of the Wood Werkman reaction in vivo* The introduction of the use of isotopes greatly facilitated the development of the work initiated by Wood and Werkman Much of their earlier chemical work has been confirmed by the use of isotopes (30, 150, 151) Isotopic carbon was fixed in the carboxyl groups of succinic acid during its formation by the propionic acid bacteria (152) It was also unexpectedly discovered that during glycerol or glucose fermentation, the propionic acid formed likewise contained carboxyl isotopic carbon (151, 29, 28, 153) It has been suggested that the formation of succinate from pyruvate occurs through the following series of reactions

$$\text{pyruvic acid} + CO_2 \rightleftharpoons \text{oxalacetic} \rightleftharpoons \text{malic} \rightleftharpoons \text{fumaric} \rightleftharpoons \text{succinic} \qquad (7)$$

Each of the reactions from oxalacetic to succinic acids has been demonstrated by Krebs and Eggleston (74) to occur reversibly in propionic acid bacteria

Further evidence for the participation of malic and fumaric acids as intermediates in this reaction has been furnished by Nishina, Endo and Nakayama (100) They have succeeded in isolating crystalline derivatives of these acids from preparations of *Bact. coli communis* fermenting glycerol and glucose in the presence of radioactive $CO_2$ When ammonium chloride was present during the fermentation, radioactive aspartic acid could be isolated, thus indicating the probability of oxalacetic acid as an intermediate

The steps concerned with the formation of propionic acid containing carboxyl

isotopic carbon derived from isotopic $CO_2$ are less certain, however The suggestion was originally made by Carson et al (30) and by Krebs and Eggleston (74) that isotopic propionic acid was formed by the reduction of isotopic pyruvic acid This pyruvic acid was considered to be formed by a reversal of the reactions leading to the formation of the symmetrical isotopic succinic acid as shown in equation (7) Werkman and Wood (145) have objected to this scheme on the grounds that according to their data every molecule of propionic acid formed would have had to originate from pyruvic acid which had first undergone the series of side reactions from oxalacetic to succinic acids They believe that it improbable that this series of reactions takes place so much faster than the direct reduction of pyruvic acid to propionic acid Werkman and Wood have suggested that, instead, propionic acid may arise from the splitting of succinic acid into propionic acid and $CO_2$ They are aware, however, that very little evidence is available for such an interpretation, for, as shown by Carson, Foster, Rubec and Barker (28), the addition of isotopic succinic acid to a preparation of propionic acid bacteria, fermenting glycerol in the absence of isotopic $CO_2$, results in the formation of very little isotopic propionic acid Conversely, upon addin isotopic propionic acid to such a fermentation, only small amounts of isotopic succinic acid could be recovered

The metabolism of $CO_2$ in several microorganisms other than the propionic acid bacteria has been studied (151, 120) Krebs (70) has made a tabulated summary of some of the microorganisms whose metabolism has been studied with the use of isotopic carbon In general, organisms which during fermentation formed the dicarboxylic acid, succinic or the monocarboxylic acid, lactic or propionic, fixed isotopic carbon of $CO_2$ This isotopic carbon without fa was always located in the carboxyl carbon atom At present, compounds of two of more carbon atoms synthesized by heterotrophic organisms and containin isotopic carbon originating from isotopic $CO_2$ are generally considered to aris by way of the Wood and Werkman reaction

$$CH_3COCOOH + CO_2 \rightarrow HOOCCH_2COCOOH \qquad (8$$

There are, however, notable exceptions to this generalization The addition of a two-carbon compound to a one-carbon compound originating from $CO_2$ (equation (3)), has been established by Lipmann and Tuttle (83) and Utter Lipmann and Werkman (135) The possible significance of this reaction in metabolism will be discussed in a later section

The early finding that heavy carbon occasionally was found in the carboxy position of acetic acid isolated from fermentations proceeding in the presence o heavy $CO_2$ indicated that $CO_2$ incorporation might occur by some mechanism hitherto undescribed (120) Recently, however, Werkman and co-workers (119 64) have been able to account for the formation of isotopic acetic acid on the basis of the Wood and Werkman reaction They considered the two possibilities that acetic acid may arise either by the oxidative decarboxylation of pyruvic acid or by the symmetrical reductive fission of succinic acid In the former case acetic acid should contain no isotope originating from $CO_2$ of the medium, in

the latter case, however, acetic acid should contain isotopic carbon in the carboxyl position since succinic acid is formed, in part at least, from pyruvic acid and isotopic $CO_2$. Their evidence indicates that both reactions occur.

It is also known that succinate may be formed by many organisms without involving the carbon dioxide fixation reaction (120). For example, the concentration of isotopic carbon in succinic acid isolated from the fermentations *Staphylococcus candidus*, *Streptococcus paracitrovorus* and *Proteus vulgaris* was below the theoretical value expected if one assumes that the entire amount of succinate is formed by the Wood and Werkman carboxylation reaction. These results coupled with other evidence give weight to Wood and Werkman's belief in the presence, in certain bacterial systems, of the reversible enzyme reaction

$$\text{succinate} + 2\,H \rightleftharpoons 2 \text{ acetate} \qquad (9)$$

Recently, with the aid of two isotopic isomers of acetic acid and with isotopic succinate, Werkman and co-workers have been able to demonstrate the presence of this reaction in at least two species of bacteria. Slade and Werkman (119) have shown that suspensions of *Aerobacter indologenes* convert carboxyl labelled acetate into isotopic succinate and vice versa. In both experiments, the isotope was located in the carboxyl position of the organic products of reaction. When acetic acid was used in which the isotopic carbon was distributed equally in the molecule, the succinate obtained contained equal amounts of isotope in the methylene and carboxyl carbons. This experiment demonstrated that the isotope in the succinic acid originated directly from acetic acid rather than indirectly from $CO_2$ of the oxidized acetate since the carboxylation reaction did not yield products containing isotopic methylene carbon. Approximately fifty per cent of the acetate formed by *Aerobacter* fermenting glucose arose by the fission of succinic acid and thirteen per cent of the total succinate by condensation of acetic acid. Thus acetate condensation and succinate fission are reactions which possess quantitative significance.

Recently Kalnitsky and Werkman (61, 62) have obtained a cell free extract of *Escherichia coli* which is capable of dissimilating pyruvate anaerobically with the formation of acetic, formic, lactic, and succinic acids. When fermentations were carried out in the presence of isotopic carbon dioxide, the last three acids contained isotopic carbon in their carboxyl groups thus demonstrating that the $CO_2$ assimilating enzymes were present in this cell free extract. The concentration of isotope in the succinic acid was too low, however, to account for the synthesis of succinic acid solely by the Wood and Werkman reaction. By experiments with acetate containing isotopic carboxyl carbon, Kalnitsky, Wood and Werkman (64) were able to demonstrate that a significant part of the succinic acid arose by another path—i.e., the condensation of acetic acid.

The rôle of $CO_2$ in metabolism has been studied in many unicellular organisms other than bacteria. The molds, *Rhizopus nigricans* and *Aspergillus niger* ferment glucose and sucrose in the presence of $C^{11}O_2$ with the formation of isotopic fumaric and citric acids, respectively (53). In both cases, isotopic carbon was found only in the carboxyl groups. van Niel et al. (99) have shown that the

holotrichous ciliate, *Tetrahymena geleii*, fermenting sugar anaerobically in th presence of radioactive $CO_2$, fixes carbon in the carboxyl position of the succin acid formed This protozoan possesses one of the most active mechanisms for th assimilation of $CO_2$ of those systems studied

The studies with isotopic $CO_2$ *in vivo* have demonstrated that the assimilatio of $CO_2$ into organic molecules is a reaction of major importance in a variety o non-photosynthetic organisms Much of the data obtained *in vivo* has bee consistent with the hypothesis that $CO_2$ assimilation proceeds by way of th Wood-Werkman reaction In order to learn more concerning the details of thi reaction, experiments have been carried out with extracts of various tissues

b *Studies of the Wood-Werkman reaction with extracts* The enzyme system involved in the Wood and Werkman carboxylation reaction have been studie by Werkman and co-workers and by Evans and his group Krampitz and Werk man (68) have isolated an enzyme from a preparation of *Micrococcus lysodeikticu* which decarboxylates oxalacetate to pyruvate in the presence of magnesium ions Cocarboxylase was not needed for the reaction Although the formation o oxalacetate from pyruvate could not be demonstrated by this bacterial prepara tion, there is evidence that it is involved in the pyruvate carboxylation reaction When oxalacetate, the enzyme and magnesium ions were allowed to react in a medium containing $C^{13}O_2$ until approximately fifty per cent of the oxalacetat disappeared, $C^{13}$ was found in the remaining oxalacetate in the carboxyl carbon c to the methylene group (145, 69)

The over-all formation of oxalacetate from pyruvate and $CO_2$ by extracts o *E coli* has been recently demonstrated by non-isotopic experiments of Kalnitsky and Werkman (63)

The rôle of phosphate in the Wood-Werkman reaction is at present unknown Krampitz et al (69) believe that oxalacetate is in equilibrium with some three-carbon acid which is more readily carboxylated than pyruvic acid They believe that the intermediate may be a phosphorylated derivative of pyruvic acid, not necessarily phosphopyruvic acid "Oxalacetate" obtained during fumarate oxidation by an enzyme preparation of *Micrococcus lysodeikticus* was considerably enriched with isotopic carbon Wood and Werkman consider that the "oxalacetate" formed by fumarate oxidation is more active, physiologically, than synthetic oxalacetate and that it undergoes decarboxylation to form the active carbon dioxide assimilating intermediate with greater facility

The crude enzyme preparation from *Micrococcus lysodeikticus* contains no enzyme which is capable of distributing the assimilated heavy carbon of oxalacetate equally on both carboxyl carbon atoms This is in contrast to the enzyme system prepared from pigeon liver by Evans, Vennesland and Slotin (49, 50) These investigators have obtained an enzyme preparation which can fix radioactive $CO_2$ in the presence of pyruvic acid through a series of reactions which result in the formation of radioactive pyruvic acid They postulate that carboxyl isotopic pyruvate is formed by a series of reactions involving carboxylation of pyruvic acid to form carboxyl isotopic oxalacetate, distribution of the isotope between both carboxyl groups and decarboxylation of this dicar-

boxylic acid to pyruvate Practically all of the $CO_2$ assimilated was present in the pyruvic acid isolated When fumarate or malate as well as pyruvate was added to the enzyme preparation in the presence of radioactive bicarbonate, a considerably greater amount of radioactivity was fixed than when pyruvate alone was the substrate The enzyme preparation contained an active fumarase system For every mole of fumarate or malate that disappeared, one mole of $CO_2$ was produced and one mole of lactic acid was formed The reaction has been divided into the following steps

$$\left.\begin{array}{l} \text{fumarate} \rightleftharpoons \text{malate} \\ \text{malate} + \text{pyruvate} \rightleftharpoons \text{lactate} + \text{oxalacetate} \\ \text{oxalacetate} \rightleftharpoons C^{14}O_2 + \text{pyruvate} \end{array}\right\} \quad (10)$$

Only 15 per cent of the total radioactivity assimilated was present in the pyruvate isolated The remainder was found in the filtrate and, in view of the lack of other intermediates in solution, was considered to be present in the lactate, malate and fumarate molecules There is evidence that the dialyzed enzyme requires manganese ions and perhaps cozymase for reactivation In contrast to the oxalacetate decarboxylase of bacterial origin, magnesium ions did not re activate the enzyme prepared from pigeon liver

3 *Carbon to carbon addition by $C_2$-$C_1$ union* At present, with certain exceptions, the Wood and Werkman reaction accounts for the incorporation of $CO_2$ into organic molecules by heterotrophic systems There have been attempts, however, to demonstrate the assimilation of $CO_2$ through addition to acetaldehyde according to the reaction

$$CO_2 + CH_3CHO \rightarrow CH_3COCOOH \quad (11)$$

but so far, no substantial evidence exists demonstrating that this reaction, an example of $C_2$-$C_1$ union, occurs to a significant extent Evans (46) has found no exchange of the carboxyl carbon of pyruvate and $CO_2$ in the presence of carboxylase even when the pressure of carbon dioxide was as high as 300 atmospheres. Carson et al (31), in a similar type of experiment, could likewise find no significant amounts of radioactive carbon in the pyruvate isolated In order to test whether, *in vivo*, any labelled pyruvate formed could be transformed or removed from the enzyme surface by a rapid reaction competing with the decarboxylation, Carson et al. have used the bacteria, *Streptococcus lactis* This species dissimilates pyruvic acid into lactic acid, acetic acid and $CO_2$ At least two separate reactions are involved

$$CH_3COCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 2H^+ + 2e^- \quad (12)$$

$$CH_3COCOOH + 2H^+ + 2e^- \rightleftharpoons CH_3CHOHCOOH \quad (13)$$

Any radioactive pyruvate formed by the reversal of reaction (12) should be trapped as lactic acid by reaction (13) However, during the course of reaction no radioactive lactic acid was formed

In spite of the failure to demonstrate the reversibility of the pyruvic acid decarboxylase reaction, Utter, Lipmann and Werkman (135) have recently reported that pyruvic acid may be formed from acetyl phosphate and formic acid ($C_2$-$C_1$ union) After incubating isotopic formic acid and non-isotopic pyruvate with extracts of *Escherichia coli*, both reactants were isolated and found to contain the same $C^{13}$ concentrations in their carboxyl positions showing that an equilibrium had been reached Other experiments by Lipmann and Tuttle (83) have demonstrated that acetyl phosphate is a component of this equilibrium Since, at the end of incubation, the $CO_2$ had a much lower concentration of isotope $C^{13}$ than did the formic acid and since the pyruvate had a high concentration of isotope, it may be concluded that the pyruvate had not been formed from a reaction of $CO_2$ and some other pyruvate precursor

Although $H_2$ and $CO_2$ were not in equilibrium with formic acid in the bacterial extracts, such an equilibrium can apparently exist in intact cells This is indicated by the formation of pyruvic acid from acetyl phosphate and $CO_2$ *in vivo*

There has been an attempt to assess the relative importance of the $C_3$-$C_1$ and $C_2$-$C_1$ reactions in accounting for the assimilation of $CO_2$ into organic molecules during certain bacterial reactions Slade et al (120) have reported the formation of lactic acid with fixed carbon in the carboxyl group by *Clostridium welchii* and by *Clostridium acetobutylicum* In spite of the formation of large amounts of lactic acid, the dicarboxylic acids were not synthesized to an appreciable extent The lack of formation of dicarboxylic acids has raised doubt about their importance as intermediates of $CO_2$ fixation in lactic acid (via reaction (7)) In a further study of this problem, Brown, Wood and Werkman (22) have measured the rate of metabolism of succinic, fumaric, malic and oxalacetic acids by preparations of *Clostridium butylicum* They have found that these acids are not metabolized at a sufficient rate to warrant their inclusion as intermediates of $CO_2$ fixation in lactic acid Instead, they believe that $C_2$-$C_1$ fixation may account for their results

The reversibility of the "phosphoroclastic" reaction is interesting because it demonstrates a second mechanism in addition to the Wood-Werkman reaction by which carbon dioxide may be incorporated into organic compounds Although the $C_2$-$C_1$ reaction undoubtedly has significance in bacterial metabolism, there is as yet no information concerning the importance of a similar reaction in mammalian metabolism Considerable evidence has accumulated pointing to the importance of the Wood-Werkman reaction in the metabolism of carbon compounds in mammalian tissues, however This will be discussed in the succeeding section

METABOLISM IN HIGHER FORMS The introduction into biological work of isotopic carbon ($C^{11}$ and $C^{13}$) has made possible new studies of mammalian intermediary metabolism both *in vitro* and *in vivo* Although deuterium is invaluable in the study of fat and protein metabolism, its use is limited when applied to the study of carbohydrate reactions since deuterium is usually lost from carbohydrates and carbohydrate precursors by oxidation or by non-specific exchange reactions During the study of carbohydrate metabolism *in vivo*, it became

evident that isotopic carbohydrate precursors are diluted by tissue constituents in much the same manner as are dietary proteins and fats Use has been made of isotopic carbon to study the interaction of these carbohydrate precursors with materials of the diluting metabolic pool

During the last eighty years, several theories (141, 102) have been proposed to explain the relationship between the foodstuffs of the diet and the protoplasmic and structural materials of the body One of the most important of these theories was developed by Folin (51) Because of the constancy of excretion of certain waste products of metabolism—e.g , creatinine—and the variability of others—e g , urea—Folin believed that there were two distinct kinds of protein metabolism occurring simultaneously which produced two different sets of waste products One type of metabolism, resulting from "wear and tear" of the biological "machinery," was characterized by the constant rate at which its products were formed and was referred to as tissue, or endogenous metabolism, the other, resulting from the introduction of foodstuffs into the organism, was characterized by the variability of the excretion of its products and was called intermediate, or exogenous metabolism According to Folin, there was little interaction between the components of these two biological systems This theory of protein metabolism was widely accepted until recent years The work of Whipple and Madden (89) and of Borsook and Keighley (16) raised questions regarding its correctness and their work has been supplemented and greatly extended by the experiments of Schoenheimer and his colleagues Using the stable isotopes of nitrogen and hydrogen, they were able to show that special proteins (116, 117, 57) and creatinine (13, 15, 14), supposedly components of an isolated endogenous system, were derived to a considerable extent from dietary sources From these and other experiments demonstrating the vast range of the interaction between foodstuffs and tissue constituents, Schoenheimer developed a new theory of metabolism The essence of Schoenheimer's theory of the dynamic state of the body constituents has been succinctly stated by Clarke (32) as follows "As a result of Schoenheimer's investigations, there has emerged a concept of metabolic 'regeneration,' wherein the central idea is the continual release and uptake of chemical substances by tissues to and from a circulating metabolic 'pool ' Coincident with these cyclic processes, there occur among the components of the pool multitudinous chemical reactions of which relatively few are concerned with elimination of waste products " Metabolic materials and reactions are, therefore, no longer to be thought of as segregated into exogenous and endogenous parts, but rather as a system whose components, whether in tissues or out, are in a state of dynamic equilibrium

It is in the light of this theory of dynamic equilibrium that the subsequent work on metabolism of organic compounds will be presented

A *Relation of Metabolic Waste Products and Essential Foodstuffs to the Metabolic Pool.* Through the isotopic study of the nitrogen waste products of metabolism, the theory of a separate exogenous and endogenous metabolism was undermined. Folin considered that urea resulted solely from the breakdown of exogenous metabolites, whereas creatinine among other substances was thought to originate

at a constant rate solely from tissue constituents In spite of the chemical relationship between creatine and creatinine, Folin (52) considered that the latter was not derived from the former, since he could not demonstrate an increase of urinary creatinine upon administering creatine Although a relationship between the content of body creatine and urinary creatinine has been subsequently established by a number of investigators (95, 108, 2), it was not until the recent work of Bloch and Schoenheimer (13, 15, 14) that the metabolism of creatine was clarified Creatinine is now known to originate solely from creatine In the rat, approximately two per cent of the body content of creatine is converted into creatinine daily The rate of creatine formation from dietary and tissue sources has likewise been found to occur at the same rate as its conversion to creatinine The experiments of Bloch and Schoenheimer have demonstrated that as much as 25 to 30 per cent of the nitrogen in the newly formed creatine is derived from the isotopic components added to the stock diet containing casein An additional part of the creatine formed is derived from the non-isotopic amino acid precursors in the diet as well as in the tissues of the animals Creatine and indirectly creatinine are thus constantly formed from food as well as from tissue material

Urea likewise is derived from both tissue and dietary constituents Ammonia, as well as the amino groups of amino acids are ready sources of the nitrogen of urea (115) $CO_2$ has been shown by the isotopic method to play a rôle in urea formation Liver slices in the presence of isotopic $CO_2$ produced urea containing high concentrations of carbon isotope (48, 107)

Whatever the biological factors may be that regulate the excretion of creatinine and permit the variability of urea excretion, they are not adequately explained by the existence of a separate endogenous and exogenous metabolism Instead, the nitrogenous end-products of metabolism are formed from a common metabolic pool, the constituents of which have been derived from both the tissues and the diet

Creatinine and urea are alike in that, once formed, they do not re-enter the metabolic reactions of the body (115) The administration of isotopic creatinine and urea results in the excretion of the compounds in the urine with unchanged isotope concentrations Because creatinine and urea once formed do not enter into metabolic reactions, one may consider that they lie outside that body of metabolites which are known collectively as the "metabolic pool"

There is still another group of substances which may be placed in this same category These are the essential foodstuffs such as the essential amino acids, essential unsaturated fatty acids, and probably many of the vitamins (4) Although during their metabolism these essential substances are undoubtedly catabolized into products which mix intimately with the large metabolic pool, they are apparently not regenerated from it The two requirements of a metabolite before it can be considered as a part of the "metabolic pool" are that it be capable of formation from and contribute to the synthesis of other components of the pool In other words, there exists among the components of the metabolic pool a state of dynamic equilibrium

B *Relation of Structural and Storage Materials to the Metabolic Pool* The extent to which the structural and storage elements of the organism react with metabolites of the metabolic pool is a function of the rate at which these materials are synthesized and degraded in the animal The structural or storage materials of the body are usually of high molecular weight and are often relatively insoluble in water Consequently, one would not expect these substances to come into complete equilibrium with the components of the metabolic pool at the same rate as do the highly reactive, water-soluble, small molecular weight compounds They will in time, however, reflect the composition of the metabolic pool from which they originate The length of time required for equilibrium to occur depends upon the individual structural or storage material Thus, mice forming fat from carbohydrate will resynthesize one-half of their total fat depots during a period of seven days (5) Consequently, if the animal maintains a static weight for seven days, one half of the original amount of fat in the depot will have been degraded and contributed to the metabolic pool in the form of oxidizable intermediates Liver fat is similarly synthesized and degraded seven times faster than is the depot fat (5), cholesterol turnover, however, is about one-third as fast (106)

It may be stated at once that, after glycogen, fat, and protein have been degraded into their simpler elements—i.e , pyruvic acid, fatty acids, and amino acids—the reactions involved in the further degradation of these substances into $CO_2$ and water have much in common (fig 1)

The mechanism of oxidation of carbohydrates was the first to be investigated. Szent-Györgyi (132) and Stare and Baumann (125) found that the addition of catalytic amounts of certain dicarboxylic acids stimulated considerably the oxidative processes of isolated tissues As a result, they assigned to these acids a rôle in the hydrogen transport mechanism of tissue oxidations Later Krebs and Johnson (76), with the knowledge that citric acid as well as $\alpha$ ketoglutaric acid acted as catalysts in oxidative reactions, postulated the formation of a six carbon, tricarboxylic acid by the condensation of oxalacetic acid with a three-carbon carbohydrate intermediate accompanied by decarboxylation The successive oxidation of citric acid, the postulated intermediate, to oxalacetic acid regenerated one of the reactants, thus explaining the catalytic effect of small amounts of dicarboxylic acids on carbohydrate oxidation The net result was the oxidation of carbohydrate to carbon dioxide and water This cycle of reactions was first called the ' citric acid cycle " It is now more commonly referred to as the "tricarboxylic acid cycle "

Certain amino acids, alanine, glutamic and aspartic acids, are closely related to components of the tricarboxylic acid cycle By transamination reactions first shown by Braunstein and Kritzmann (19), these three acids may be transformed into their respective keto acids pyruvic, $\alpha$-ketoglutaric and oxalacetic For example

$$CH_3\ CO\ COOH + HOOC\ CH_2\ CH_2\ CHNH_2\ COOH \rightleftharpoons CH_3\ CHNH_2\ COOH + HOOC\ CH_2\ CH_2\ CO\ COOH \quad (14)$$

Since these reactions are reversible, acids of the tricarboxylic acid cycle can either be formed from or contribute to the synthesis of certain amino acids In addition to enzymes of the transamination system, there is an l-amino acid oxidase which reversibly converts glutamic acid into $\alpha$-ketoglutaric acid and ammonia (44)

Recently it has been demonstrated that the organic acids of the tricarboxylic acid cycle are intermediates in the oxidation of fatty acids and ketone bodies (20, 21, 146, 26, 105)

The organic acids of the tricarboxylic acid cycle are consequently the meeting point of protein, fat and carbohydrate metabolism Since the conversion of one foodstuff into another may involve reactions of the tricarboxylic acid cycle, a substance may be diluted by the components of the cycle during the course of the reaction Even though the tricarboxylic acid cycle may not be directly concerned in a given reaction of the organism, the reacting substance may come into equilibrium with the components of the cycle before undergoing its primary transformation Thus, the composition of the primary product of reaction will reflect the secondary circuitous equilibrium reactions involving the reactant

1 *The tricarboxylic acid cycle and the rôle of* $CO_2$ The mechanism of the oxidative dissimilation of carbohydrates has been studied by Krebs and Eggleston (72) using pigeon breast muscle As mentioned previously, they found that the presence of a small amount of dicarboxylic acid was an essential component of the system which oxidizes pyruvic acid Since only small amounts of oxalacetic acid are necessary to effect the oxidation of large amounts of pyruvic acid, oxalacetate must be continually regenerated by the cyclic reactions by which pyruvic acid is oxidized The addition of malonic acid, a specific inhibitor of the succinoxidase system, prevents the regeneration of oxalacetic acid and causes the accumulation of the products of the condensation of pyruvic and oxalacetic acids—i e , citric, $\alpha$-ketoglutaric and succinic acids When a tissue, poisoned with malonate, uses up its endogenous sources of oxalacetate, the oxidation of pyruvate stops and can only be started again by the addition of oxalacetate or its equivalent The amount of pyruvate disappearing under these circumstances is equivalent to the amount of oxalacetate added to the tissue

In contrast to pigeon muscle mince, Evans (45) has found that pigeon liver mince poisoned with malonate does not require the addition of dicarboxylic acids to oxidize pyruvic acid Apparently, pigeon liver mince is capable of synthesizing its own dicarboxylic acids from pyruvate Krebs and Eggleston (73), from indirect evidence, suggested that the dicarboxylic acids arose from pyruvate by means of the carboxylation reaction of Wood and Werkman (equation (8)) In their experiments with normal pigeon liver mince, a decrease of utilization of pyruvate and consequently of dicarboxylic acid production was observed when bicarbonate was excluded from the incubating medium Thiamine was also demonstrated to play a rôle in pyruvate dissimilation The rate of pyruvate dissimilation was decreased in liver suspensions of thiamine-deficient pigeons containing an adequate amount of bicarbonate The addition of thiamine to these suspensions increased the rate of production of dicarboxylic acids several fold

Direct evidence for the utilization of carbon dioxide by rat liver was obtained by Ruben and Kamen (111) using radioactive carbon Although assimilation of carbon dioxide was demonstrated in their experiments, no specific reaction products containing radioactive carbon were isolated

However, Evans and Slotin (47), extending their earlier experiments, isolated radioactive $\alpha$-ketoglutaric acid formed by pigeon liver mince from pyruvate in

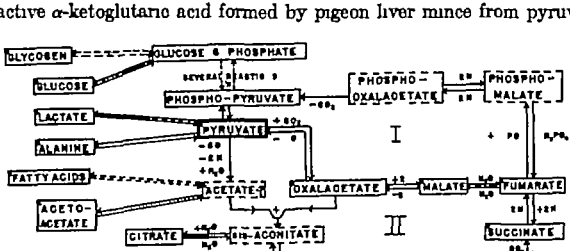



Fig 1 Diagram illustrating reactions involving examples from carbohydrate, protein and fat metabolic pools

*Cycle I* Gives hypothetical reactions of pyruvate and $CO_2$ in formation of phospho pyruvate

*Cycle II* Gives reactions of tricarboxylic acid cycle in oxidation of pyruvate to $3CO_2 + 2H_2O$

Compounds marked * have been studied by isotopic analysis

Broken boxes designate substances of hypothetical significance

Broken lines designate reactions omitted.

Formulas of acids of cycles I and II are to be found in figure 3 except

| | |
|---|---|
| Succinic | HOOC $CH_2$ $CH_2$ COOH |
| Cis-aconitic | HOOC CH C $CH_2$ COOH<br>COOH |
| Isocitric | HOOC CHOH CH $CH_2$ COOH<br>COOH |
| Citric | HOOC $CH_2$ COH $CH_2$ COOH<br>COOH |

the presence of radioactive bicarbonate The $\alpha$-ketoglutarate isolated contained radioactivity only in the carboxyl carbon $\alpha$ to the keto group According to Krebs' scheme for the dissimilation of pyruvic acid, one molecule of pyruvate condenses with one molecule of oxalacetate to form the hypothetical intermediate, oxalcitraconate By oxidative decarboxylation, this latter compound may be converted into citric acid As citric acid is a symmetrical compound, the $\alpha$ ketoglutaric acid formed from citric acid might have been expected to

contain radioactive carbon in either of its carboxyl groups However, it did not The observations of Evans et al indicate, therefore, that citric acid cannot be a direct intermediate in the formation of $\alpha$-ketoglutaric acid from pyruvic acid by liver mince Furthermore, the specific radioactivity of the $\alpha$-ketoglutarate formed was not altered by the addition of citrate to the medium If citrate were an intermediate in the dissimilation of pyruvate, the specific radioactivity of the $\alpha$-ketoglutarate formed should have been considerably reduced

Several other interesting observations have been made by Evans and Slotin (47) Alpha-ketoglutarate formed in the presence of fumarate, pyruvate and radioactive carbon dioxide in pigeon muscle mince does not contain radioactive carbon, thus indicating that the carboxylation reaction (equation (8)) does not take place in this muscle preparation This experiment is also indirect evidence that the assimilation of carbon dioxide by liver is not a non-specific exchange phenomenon, but rather due to a specific chemical reaction That the products of carbon dioxide assimilation may include amino acids is indicated by the observation that considerable radioactivity was released by treating the liver mince with either chloramine T or ninhydrin

Wood, Werkman, Hemingway and Nier (154) have confirmed and extended the evidence obtained by Evans and Slotin They have studied the aerobic formation of succinate from pyruvate by pigeon liver mince in the presence of malonate and its synthesis anaerobically in the absence of inhibitor In the former case, the succinate isolated did not contain isotopic carbon derived from the isotopic $CO_2$ present in the medium As in the experiments of Evans and Slotin, the $\alpha$-ketoglutarate contained excess isotopic carbon in the carboxyl group $\alpha$ to the keto group Fumarate and malate both contained isotopic carbon in the carboxyl position Since succinate contained no isotopic carbon, it was considered to arise solely from the oxidative decarboxylation of $\alpha$-ketoglutarate This experiment demonstrated conclusively that malonate in the concentrations used does completely inhibit succinic dehydrogenase and hence prevents the formation of succinic acid by the reduction of fumaric acid As expected, in anaerobic experiments in the absence of malonate, the succinate as well as the fumarate and malate contained excess isotopic carbon in the carboxyl position

These experiments have demonstrated that there are at least two pathways for the formation of succinate from pyruvate by pigeon liver mince (fig 1) By one path, succinate is formed by reductive steps from oxalacetate, the latter being synthesized from pyruvate and carbon dioxide according to the Wood and Werkman reaction The last step in this series—i e , fumarate $\rightleftharpoons$ succinate—is completely inhibited by malonate The other path for succinate formation is aerobic There are no enzymes in this series of oxidative reactions which are specifically inhibited by malonate According to Wood at al , the first step of this series is an oxidative condensation of oxalacetate and pyruvate to form cisaconitate This latter compound is hydrated to isocitrate which is oxidized and decarboxylated to $\alpha$-ketoglutarate The place of isocitrate and cisaconitate in this scheme is at present tentative Evans and Slotin believe that cisaconitate cannot be the intermediate in this series of reactions since citrate, isocitrate and

cisaconitate are in equilibrium with each other Krebs (71), on the other hand, postulates that the rate of conversion of cisaconitate to $\alpha$ ketoglutarate is so much faster than its conversion to citrate that equilibrium between cisaconitate and citrate never occurs, at least, in pigeon liver

Ochoa and Weisz-Tabori have recently demonstrated the presence of oxalosuccinic carboxylase in pig heart preparations (100a) This observation has led Ochoa to the conclusion that the enzyme system composed of aconitase, isocitric dehydrogenase and oxalosuccinic carboxylase may provide another important manner in which $CO_2$ is utilized (100b)

Thus, it may be stated that although the tricarboxylic acid cycle provides a reasonable and logical description of the chemical reactions concerned with the conversion of pyruvate to $CO_2$ and water, and although there exists considerable evidence of an indirect nature pointing to its significance *in vitro*, direct proof for its correctness in detail and the magnitude of the rôle it plays *in vivo* await further data

2 *Glycogen synthesis and glucose metabolism* There are two fortunate factors which contribute greatly to the isotopic study of the synthesis of liver glycogen from its precursors One of these is the rapidity with which glycogen formation takes place in the liver after the administration of a variety of glycogenic substances The other is the fact that the preformed stores of liver glycogen can be reduced to a very low amount by fasting, so that the composition of the glycogen isolated after the administration of an isotopic glycogenic substance directly reflects the composition of the compounds from which it originates

a *Reactions of isotopic $CO_2$ and lactate in liver glycogen synthesis* During the course of experiments, investigating the sources of glycogen carbon, Solomon, Vennesland, Klemperer, Buchanan and Hastings (121) observed that starved rats fed non isotopic lactic acid and injected intraperitoneally with isotopic sodium bicarbonate, ($NaHC^{*}O_3$), had an appreciable quantity of radioactive carbon in the glycogen which was formed in the liver [2] From the specific radioactivity of the expired $CO_2$, and the specific radioactivity of the newly formed glycogen carbon, the proportion of this glycogen carbon which had been $CO_2$ in the body was estimated and found to average 11 per cent In three out of the seven experiments reported, the proportion was 13 9, 14.2, and 15 6 per cent These results were interpreted as being consistent with the reactions suggested

[2] It should be recorded that at a conference of the Harvard investigators early in the fall of 1939 our former colleague Dr Birgit Vennesland had with infallible feminine intuition suggested the possibility of $CO_2$ incorporation in glycogen, and proposed an experiment with radioactive $CO_2$ and non radioactive lactate This suggestion seemed fantastic to the undersigned and it was not carried out until the spring of 1940 And even then it was performed as a control experiment to insure the adequacy of the experimental procedures employed when radioactive lactic acid was fed

Finding radioactivity in the liver glycogen whose source was $CO_2$ continued to be viewed with skepticism until Doctor Vennesland succeeded after several disappointments in winning the race against the five hour deadline set by the rapidly decaying $C^{11}$ and demonstrated the presence of the radioactivity in the osazone isolated from hydrolyzed glycogen (137) A B H

by Lipmann (82) and Kalckar (60) for the formation of phosphopyruvate from dicarboxylic acids (fig 2) According to this hypothesis, the reactions tracing the fate of isotopic carbon atoms starting with the Wood-Werkman reaction (pyruvate + $CO_2$) are given in figure 2 It is to be noted that these reactions

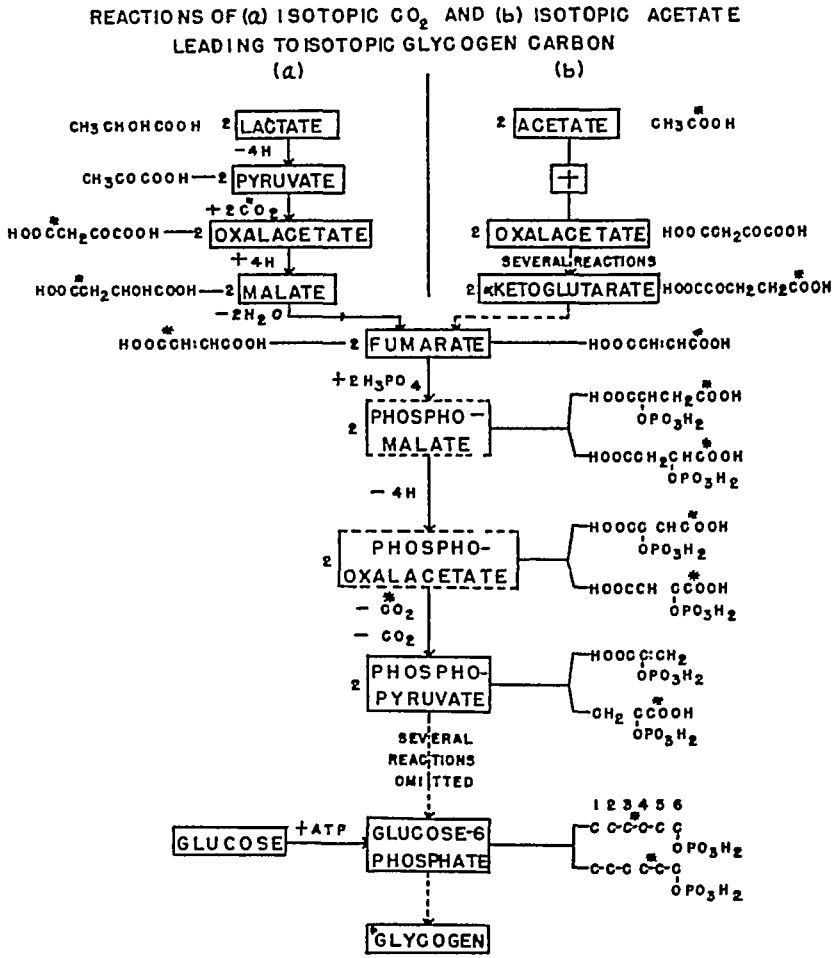



Fig 2 The positions of isotopic carbon in glycogen intermediates are indicated by *
Reactions proposed for incorporation of isotopic carbon in liver glycogen *in vivo* (a) after $C^*O_2$ and lactate administration (Ref 47, 154, 121), after $C^*O_2$ and glucose administration (Ref 137, 147), (b) after isotopic acetate, $CH_3C^*OOH$, and glucose (Ref 81, 26) Reactions of $C^*O_2$ are shown at left, reactions of isotopic acetate at right Isotopic carbons from both sources are indicated as having identical positions from fumarate to glycogen
Broken boxes designate hypothetical compounds, broken lines designate omitted reactions Only the carbons of glucose-6 phosphate have been indicated

would predict that one-sixth of the carbon atoms of glycogen were derived from $CO_2$ carbon,

Recent experiments with non-isotopic glucose and with isotopic $C^{13}O_2$ by Wood, Lifson and Lorber (147) have proved that the $CO_2$ carbon occupies position 3 or 4 in the glucose derived from the newly formed glycogen

From work recently reported by Lardy and Ziegler (77), it would appear that direct phosphorylation of pyruvate to form phosphopyruvate can occur in extracts of rat muscle in the presence of potassium ions Previous attempts to demonstrate the direct phosphorylation of pyruvate had been unsuccessful (94) and had led to the hypothesis of Lipmann and of Kalckar referred to above The observations of Lardy and Ziegler taken together with the experiments of Evans, Vennesland and Slotin (50) and of Wood, Vennesland and Evans (147a) present an alternate pathway whereby isotopic carbon from $C^*O_2$ could participate in glycogen formation. (See fig 1 and the following reactions )

$$\left.\begin{array}{l} \text{pyruvate} + C^*O_2 \rightleftharpoons \text{oxalacetate}^* \\ \text{oxalacetate}^* \rightleftharpoons \text{pyruvate}^* + CO_2 \\ \text{pyruvate}^* + ATP \rightleftharpoons \text{phosphopyruvate}^* + ADP \end{array}\right\} \quad (15)$$

Until further work on this question has been carried out, it is not possible to evaluate the relative biological importance of the two ways whereby phosphopyruvate may be formed *in vivo*

b *Reactions of isotopic lactate in liver glycogen synthesis* Although the synthesis of liver glycogen is known to be increased by lactate administration (34), the extent to which the lactate molecules administered comprise the glucose units of the glycogen was unknown prior to the use of isotopic lactate Experiments have now been reported using isotopic lactate with radioactive carboxyl carbon ($CH_3CHOHC^{11}OOH$) (37, 33), and with radioactive $\alpha$ or $\beta$ carbons ($CH_3C^{11}$ HOHCOOH or $C^{11}H_3CHOHCOOH$), (136) which clarify this question

It was found that in rats fed isotopic $\alpha$-$\beta$ carbon lactate an average of 16 per cent of the glycogen formed was derived from three-carbon lactate units after allowing for the radioactivity due to $CO_2$ incorporation This may be interpreted as indicating a six-fold dilution of the lactate molecules in the effective carbohydrate metabolic pool The reactions which would account for the presence and positions of the radioactive carbons in the glucose units of the glycogen are indicated in the left hand column of figure 3

These reactions would predict the presence of isotopic carbon in positions 1–6, 1–5, 2–6, or 2–5 of the glucose unit The correctness of this prediction of the positions of the isotopic carbon has not been proven

In the experiments with isotopic carboxyl lactate, the specific radioactivity of the glycogen formed was considerably less than in the experiments with isotopic $\alpha$-$\beta$ carbon lactate It would appear from the data presented that an average of only 6 per cent of the glycogen was derived from three-carbon lactate units after allowing for the radioactivity due to $CO_2$ incorporation However, when the reactions concerned in the conversion of isotopic carboxyl lactate are reviewed (fig 3, right-hand column), it is seen that only half as much isotope per glucose unit would be expected as in the experiments with $\alpha$-$\beta$ lactate This is due to the loss of radioactive carbon as $CO_2$ from half the original three-carbon lactate units Since the reactions listed in figure 3 predict that twice as much isotopic carbon is to be expected in glycogen derived from isotopic $\alpha$-$\beta$ carbon lactate as from isotopic carboxyl carbon lactate, and since 16 per cent and 6 per

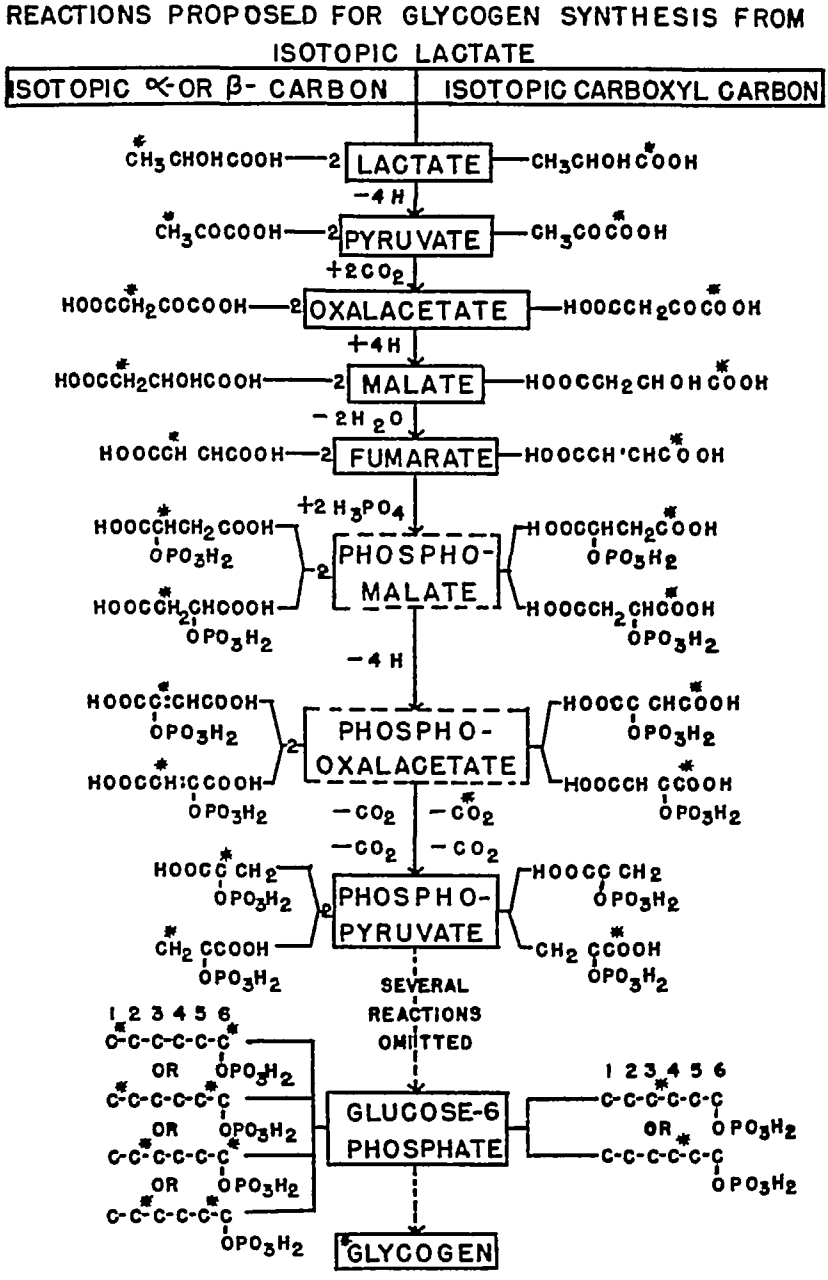



Fig 3 The positions of isotopic carbon in glycogen intermediates are indicated by * Reactions of isotopic $\beta$ carbon lactate only shown at left Reactions of isotopic $\alpha$ carbon lactate would give same end result and have been omitted (Ref 136)

Reactions of isotopic carboxyl carbon shown at right (Ref 33)

Broken boxes designate hypothetical compounds, broken lines designate omitted reactions Only the carbons of glucose-6-phosphate molecules have been indicated

cent, respectively, were found, support is given to the over all theory of $CO_2$ assimilation during glycogen synthesis The correctness of the details of reac tions listed in figure 3 and the positions occupied by the isotopic carbon in the glucose units await further study

c *Reactions of isotopic $CO_2$ and glucose in liver glycogen synthesis* Experiments have also been carried out in which glucose (137) was fed to a fasted animal and radioactive bicarbonate injected Approximately 13 per cent of the carbon of the newly formed glycogen was found to have originated from carbon dioxide This is in good agreement with the amount of carbon dioxide carbon entering glycogen during its formation from lactate

During the synthesis of glycogen from glucose *in vitro* by rabbit liver slices, however, only small amounts of radioactive carbon dioxide were incorporated into the glycogen formed (137) This is in contrast to the results obtained *in vitro* with radioactive $NaHC^{11}O_3$ and non isotopic pyruvate as substrate in- stead of glucose Approximately 12 per cent of the glycogen formed *in vitro* from pyruvate by rabbit liver slices originated from carbon dioxide (24) In experiments, as yet unpublished (23) in which non isotopic pyruvate was fed to rats and isotopic $NaHC^{11}O_3$ was injected, glycogen was formed, *in vivo*, which contained 14 per cent carbon originating from $CO_2$

The agreement between the results obtained with pyruvate *in vivo* and *in vitro* and the disagreement obtained in the case of glucose is of interest in considering the metabolism of glucose in the body According to the present theories of the formation of glycogen from glucose developed from the work by Cori and his co-workers using pure enzyme systems, no step would appear to require carbon dioxide incorporation (35)

$$\text{glucose} \rightarrow \text{glucose-6-phosphate} \rightarrow \text{glucose-1-phosphate} \rightarrow \text{glycogen} \qquad (16)$$

That this is probably the case during glycogenesis by a tissue system in which dilution and equilibrium phenomena play a minor rôle is indicated by the *in vitro* experiments with rabbit liver slices In the intact animal, however, absorbed glucose is subject to many reactions which do not occur in the isolated system. The fact that the radioactive carbon of injected $NaHC^{11}O_3$ is incorporated into glycogen to the same extent after glucose feeding as after lactate and pyruvate feeding indicates that the glycogen deposited has originated in part, at least, from compounds which have undergone a carbon dioxide incorporation reaction Since the glucose absorbed must have represented a significant portion of the final metabolic pool, it must be assumed that the ingested glucose comes into equilibrium very rapidly with the dicarboxylic acid pool before its conversion to glycogen Thus may occur according to the reactions shown in figure 1 The reactions between glucose-6-phosphate and the dicarboxylic acids and pyruvate are assumed to occur much more rapidly than the conversion of glucose-6-phos phate to glycogen.

As stated in the preceding section, Wood, Lifson and Lorber (147), using $C^{13}$, have provided further impressive evidence for the validity of the above reactions for $CO_2$ assimilation into glycogen following the administration of glucose to

white rats By chemical and bacteriological procedures, these investigators have found that $C^{13}$ is located in the third or fourth carbon atoms of the glucose obtained from the liver glycogen of rats receiving glucose and isotopic sodium bicarbonate These results would have been expected if glycogen is synthesized from three-carbon units by way of the dicarboxylic acids and the classical glycolytic scheme (fig 2)

Although glucose plays an active rôle in the equilibrium reactions of the metabolic pool, glycogen is relatively inert in this respect When radioactive bicarbonate was injected into a well-fed rat with large stores of liver glycogen, no radioactivity was found in the liver glycogen isolated after two and one-half hours Thus, the incorporation of $C_{11}$ in liver glycogen occurs to an appreciable extent only when the animal is actually making and depositing glycogen (121)

d *Glycogen synthesis in muscle* A study has also been made of glycogen synthesis in muscle tissue after feeding lactate, pyruvate or glucose and injecting $NaHC^{11}O_3$ After the administration of glucose and $NaHC^{11}O_3$, the muscle glycogen contained significant amounts of $C^{11}$ (137) Such was not the case after feeding lactate or pyruvate (121, 23) Whether or not new muscle glycogen was formed in these experiments is not known

On the other hand, Boxer and Stetten (18), using the presence in glycogen of deuterium, after the administration of $D_2O$, as a measure of glycogen synthesis, found an appreciable formation of muscle glycogen 4 hours after the administration of lactate to a fasted rat The failure of $C^{11}$ to appear in muscle glycogen after the administration of lactate may have been due to lack of glycogen synthesis in this tissue during the experiments cited above or to a difference in the mechanism of glycogen synthesis in muscle and liver It is known, however, that $CO_2$ may be incorporated into glycogen during its synthesis by muscle tissue This has been shown by the experiments of Lorber, Hemingway and Nier (84), who have studied the formation of muscle glycogen in an isolated cat heart perfused with a solution containing glucose or lactic acid and isotopic carbon dioxide Regardless of the nature of the substrate, the glycogen isolated from the heart muscle contained an excess of heavy carbon By assuming that each isotopic hexose unit of the glycogen contained only one isotopic carbon atom, Lorber et al have calculated that, on an average, one-sixth of the total glycogen isolated after an experimental period of 1 5 hours was formed by reactions involving the incorporation of carbon dioxide

It is entirely possible that two paths of glycogen synthesis from lactate occur, the one supplementary to the other In certain tissues such as muscle, where glycogen synthesis from lactate does not take place at a rapid rate and where $CO_2$ assimilation reactions are not particularly marked, the predominant pathway of glycogen formation may be by the direct conversion of pyruvate to phosphopyruvate In contrast, in liver tissue, where glycogen synthesis from lactate does take place at a rapid rate, glycogen formation involving the synthesis of dicarboxylic acids and the incorporation of $CO_2$ may be the primary pathway

e *Nutritional factors and glucose metabolism* Ussing (134) demonstrated that stably-bound deuterium is incorporated into liver glycogen during its synthesis

from glucose by rats injected with deuterio-water Since the direct conversion of glucose to glycogen according to equation (16) should not involve the incorporation of stably bound deuterium into the glycogen molecule, Ussing's results have been interpreted as meaning that part of the glycogen was synthesized from carbon units smaller than glucose From the reactions of figure 2, it would be theoretically possible for all of the hydrogen atoms of a three-carbon glycogen precursor such as lactic acid to be removed or replaced by deuterium during its conversion to glycogen By feeding lactate to fasted rats injected with deuterio-water, Boxer and Stetten (18) have demonstrated that deuterium of deuterio-water is incorporated into practically all the positions of the glycogen molecule theoretically possible during its synthesis from this three-carbon precursor In subsequent experiments in which glucose and deuterio-water were fed to animals and glycogen isolated under a variety of experimental conditions, these investigators have assumed that glycogen containing deuterium is formed by reactions involving small carbon chains, whereas glycogen containing no excess of deuterium results from the direct conversion of dietary glucose to glycogen by way of reactions of equation (16) By comparing the deuterium concentration of newly formed glycogen with the deuterium concentration of the body water in which it was formed, Boxer and Stetten have estimated in their experiments the proportion of glycogen synthesized by each of these two pathways

When deuterio-water was administered to well nourished animals on a high carbohydrate diet, the deuterium concentration of the liver glycogen gradually increased until it reached a constant level from 8 to 16 days after the beginning of the experiments (127) At this "saturation level" it was estimated that 43 per cent of the newly formed liver glycogen was derived by reactions which incorporated deuterium—i.e , by synthesis from precursors with carbon chains smaller than glucose Only 3 per cent of the total glucose metabolized was converted to glycogen, demonstrating that the conversion of glucose to glycogen is not a major pathway of glucose metabolism Under certain conditions, the metabolism of the animal on a high carbohydrate diet could be altered so that a greater percentage of newly formed glycogen originated from carbon chains smaller than glucose These conditions were (a) the administration of adrenalin (129), (b) the administration of alloxan (128), (c) thiamine deficiency (17), or (d) fasting of the animal 24 hours prior to the feeding of glucose (18) In each of these experiments, the isolated glycogen contained a greater deuterium concentration than a sample isolated from a normal, well-nourished control In contrast, the administration of glucose and insulin to a fasted animal resulted in the formation of glycogen predominantly by the union of hexose units The results with adrenalin confirm earlier experiments of Cori and Cori (36) who have stated that glycogen deposited in the liver of the previously fasted rat in response to adrenalin was derived from blood lactate

Deuterium of deuterio-water is also incorporated into fatty acids in constant amounts during their synthesis from glucose (115) Using this as a measure of fat synthesis from glucose, Stetten and Boxer have studied the rate of fat synthesis after the feeding of glucose to animals under some of the various experi-

mental conditions listed above Thirty per cent of the daily glucose supplements of the diet of a well-nourished animal is converted into fatty acids (127) When these animals were poisoned with alloxan, thus producing an insulin deficiency, the rate of fatty acid synthesis was reduced to 5 per cent of the normal value (128) This blocking of fat synthesis in hypoinsulinism thus represents the cessation of a major pathway of carbohydrate utilization No such depression of fatty acid synthesis was observed in the case of the phlorhizinized animals where the islet tissue is intact (129)

The rate of fatty acid synthesis from glucose was also greatly diminished in the thiamine-deficient animal (17) In another group of normal animals whose caloric intake was lowered to that of the thiamine-deficient group, the synthesis of fatty acids was likewise hindered Boxer and Stetten, consequently, consider that the lack of synthesis of fatty acids in the thiamine-deficient animal resulted from inanition rather than from any specific effect of the thiamine deficiency on carbohydrate metabolism

3 *Metabolism of fatty acids* Although much has been learned concerning the metabolism of carbohydrates, there has been until recently but little conclusive experimental evidence available on the intermediates of fatty acid and ketone body oxidation In the present section, this new evidence gained from isotopic studies will be presented and its interpretation in terms of current theories of fat metabolism will be discussed

a *Formation of ketone bodies* The classical experiments of Knoop (66) and Dakin (38) were largely responsible for establishing the theory of successive $\beta$-oxidation of fatty acids According to this theory, two-carbon compounds are successively split off from fatty acids and oxidized Hurtley (58) in 1916 was the first to raise objection to this scheme of oxidation of fatty acids He proposed the theory of multiple alternate oxidation to account for the absence of short chained fatty acids in the urine of diabetics excreting excessive amounts of ketone bodies According to this hypothesis, approximately four ketones are produced by the oxidation of one sixteen-carbon fatty acid, instead of one which would be expected from the theory of successive $\beta$-oxidation This point of view has been extended by work of Deuel (27), Stadie (122), Blixenkrone-Møller (9), Quastel (59) and their associates Deuel and co-workers (27) have found that the administration of fatty acids with chains of four to ten carbon atoms resulted in the excretion of ketone bodies equal to or considerably more than the amount excreted in the urine after feeding equimolecular amounts of acetoacetic acid Blixenkrone-Møller, measuring the ketone production and oxygen consumption of perfused diabetic livers, came to the conclusion that the low ratio of oxygen consumed to ketones produced could not be accounted for by the theory of $\beta$-oxidation Stadie, Zapp, and Lukens (123) have extended Blixenkrone-Møller's experiments By measurement of oxygen consumption, carbon dioxide and urea production of liver slices, they have been able to estimate the amount of oxygen that the tissue used in the production of the ketone bodies found About 1 11 moles of ketone bodies were produced for every mole of oxygen disappearing The theory of multiple alternate oxidation requires

a ratio of oxygen consumed to ketones produced of 1.25, the theory of β-oxidation, 6.5.

In spite of these excellent quantitative experiments, it has been generally recognized that β-oxidation does take place in the organism Schoenheimer and Rittenberg (118) found that deuterio palmitic acid was deposited in the depots of animals fed deuterio stearic acid. Likewise, animals receiving deuterio palmitic acid were capable of synthesizing deuterio stearic acid (130) Such experiments provided substantial evidence in support of the theory of successive β-oxidation of fatty acids

From these diverse and conflicting data has evolved a theory of fat oxidation which represents a compromise between the two theories referred to above According to MacKay and co-workers (86), fatty acids undergo successive β-oxidation forming two-carbon compounds which immediately condense to produce a four-carbon ketone body This hypothesis then embodies the mechanism of oxidation of fatty acids suggested by Knoop, but is more complete than the older theory since it accounts for the accumulation of ketone bodies during fat metabolism Moreover, this theory explains certain findings which cannot be reconciled with the theory of multiple alternate oxidation—namely, the formation of more ketone bodies from hexanoic than from butyric acid (41) and the formation of ketone bodies from valeric acid, three carbon atoms of which are known to form glycogen (79, 87)

The recent study of the oxidation of carboxyl labelled fatty acids to acetoacetate by rat liver slices has rendered considerable support to MacKay's theory of β-oxidation-condensation Wemhouse, Medes and Floyd (143) have found that acetoacetate formed from the oxidation of carboxyl labelled octanoic acid contained isotopic carbon distributed equally in the carboxyl and carbonyl carbons The theory of β-oxidation-condensation predicts such an equal distribution of isotope Acetoacetate produced from carboxyl tagged octanoic acid by a process of multiple alternate oxidation should theoretically contain isotope only in the carboxyl position The classical successive β-oxidation theory of Knoop would predict the formation of non isotopic acetoacetate from carboxyl labelled octanoic acid

Using isotopic carboxyl butyrate as substrate in a similar experiment, Medes, Wemhouse and Floyd (91) found that the acetoacetate produced, likewise contained heavy carbon in the carbonyl and carboxyl positions. In this case, however, the carboxyl carbon atom contained a significantly higher concentration of isotope than the carbonyl carbon This unequal distribution of isotope in the case of butyric acid cannot be interpreted in terms of the theory of multiple alternate oxidation By definition, multiple alternate oxidation involves the simultaneous oxidation of alternate carbon atoms with cleavage of a carbon to-carbon bond at each fourth carbon atom The incomplete distribution of isotope in this case does demonstrate, however, that the formation of the β-keto acid may precede the carbon-to-carbon cleavage

Although the results of these authors (143) demonstrate that two-carbon fragments are formed and condense in the process of octanoic acid oxidation, there is still doubt concerning the exact mechanism of this oxidation It is possible,

for instance, that acetoacetate is formed from octanoic acid by multiple alternate oxidation and that the distribution of the isotopic carbon between the carbonyl and carboxyl carbons results from a cleaving and resynthesis of acetoacetate itself

The problem of the synthesis of ketone bodies from acetic acid has been studied with carbon isotopes both *in vivo* and *in vitro* In confirmation of the experiments of MacKay and his co-workers (86), Swenseid, Barnes, Hemingway and Nier (131) have shown that ketone bodies formed by rats after the administration of isotopic acetate contained $C^{13}$ (fig 1)

Medes, Weinhouse and Floyd have studied the formation of acetoacetic acid from carboxyl labelled acetic acid by a variety of tissues *in vitro* In liver slices (92), acetic acid is metabolized with an accumulation of acetoacetic acid The latter compound, when derived from isotopic acetic acid, contained $C^{13}$ in both carboxyl and carbonyl positions A slight excess was found in the carboxyl position indicating that the $\beta$ and $\gamma$ carbon atoms of acetoacetate may have been derived in part from an endogenous source Kidney and heart slices oxidized acetate without the accumulation of ketone bodies If non-isotopic acetoacetate was added to kidney slices, however, during the oxidation of isotopic acetate, isotopic acetoacetate could be isolated (144) This was not true of heart tissue (93) Medes, Weinhouse and Floyd have interpreted their data as indicating that two pathways for acetate utilization exist in animal tissues

From the information so far available, it would appear that, as proposed by MacKay, fatty acids are metabolized by $\beta$-oxidation into a two-carbon intermediate which may condense to form acetoacetate Those two-carbon units which do not condense in this manner may undergo a variety of reactions They may be oxidized to $CO_2$, used in the synthesis of cholesterol or fatty acids, or act as acetylating agents Other isotopic evidence to follow is concerned with the rôle of a two-carbon compound in these reactions and emphasizes the importance of this compound in metabolism

b *Sources and reactions of acetate* As previously mentioned, the early experiment of Schoenheimer and Rittenberg (118) demonstrated that a two-carbon unit is split from stearic acid forming palmitic acid In a reverse manner, palmitic acid may yield stearic acid (130) The incorporation of deuterium along the entire fatty acid chain by animals synthesizing fat from carbohydrate in the presence of deuterium is further evidence that this synthesis involves the condensation of a number of short chained compounds (115) Recently, Rittenberg and Bloch (104) have shown that isotopic acetic acid may contribute its carbon atoms to the synthesis of fatty acids Acetic acid labelled in the methyl group with deuterium and in the carboxyl group with heavy carbon was administered to rats and mice for a period of eight days The fat isolated from their tissues contained both deuterium and heavy carbon in excess of the $D_2O$ and $C^{13}O_2$ concentrations of the fluids The finding that the $C^{13}$ concentration of the carboxyl carbon atoms of the saturated fatty acids was approximately twice that of the $C^{13}$ concentration in all the carbon atoms of the total molecule suggested that the fatty acids are synthesized by successive condensations of two-carbon units

The conversion of acetic acid to cholesterol has been studied by Bloch and

Rittenberg (11) in considerable detail Cholesterol isolated after the feeding of deuterio acetate contained deuterium in both the nucleus and side chain, thus indicating that the synthesis of cholesterol involves the condensation of small carbon units, either acetate itself or a derivative of acetate In a search for the identity of this cholesterol precursor, Bloch and Rittenberg have fed a variety of deuterio-marked compounds In no instance have any of the deuterio substituted compounds administered under comparable conditions resulted in higher deuterium concentration in the isolated cholesterol than after feeding deuterio acetate itself These substances are believed to participate in cholesterol formation by virtue of the fact that they yield acetate during the course of their catabolism

Although the synthesis of deuterio cholesterol has been used as a measure of the ability of certain deuterium-containing compounds to yield deuterio acetate, Bloch and Rittenberg (12, 10) have recently studied the acetylation of phenyl amino butyric acid in this respect Their work on this subject was based on the original finding of Bernhard (3) that deuterio acetyl derivatives were excreted when a foreign amine and deuterio acetic acid were administered to dogs In order to compare the conversion to acetyl of various substances containing different concentrations of deuterium and administered in different doses, Bloch and Rittenberg have compared their results in terms of their coefficients of utilization. The acetylating agent itself should have the highest coefficient of utilization The administration of deuterium labelled acetate, ethyl alcohol, alanine, $\beta,\gamma$-dideuterio butyrate, ethyl deuterio myristate, deuterio dl leucine, deuterio isovalerate, $\alpha,\beta$-dideuterio butyrate and deuterio n valerate yielded deuterium-containing acetyl groups in the order named The acetyl group of l-acetyl phenyl amino butyric acid excreted after the feeding of deuterio propionate, succinate, valine, isobutyrate, ethyl 10,11 dideuterio undecylate and ethyl 9,10 dideuterio stearate contained no deuterium The accuracy of the value assigned to the coefficient of utilization depends upon the amount of the administered material catabolized and the extent to which deuterium is removed from the carbon atom through oxidation and labilization reactions Thus, deuterium on the $\alpha$ carbon of acetoacetic acid is somewhat more labile than that of the $\omega$ carbon Because of this labilization of deuterium on the $\alpha$ carbon atom of a $\beta$ keto acid, very little deuterium is found in the acetyl group of the excreted acetyl phenyl amino butyric acid after administration of deuterio fatty acids. However, as expected, deuterium in the $\omega$ position of ethyl deuterio myristate appears in the acetyl group

Since deuterium in the $\beta$ carbon of alanine is readily converted into the acetyl group, it is assumed that two carbons of pyruvic acid are available for acetylation reactions The finding that deuterium of $\alpha,\beta$-dideuterio propionic acid was not incorporated into the acetyl group of acetyl phenyl amino butyric acid indicates that pyruvic acid is not an intermediate in the oxidation of propionic acid, as has been long supposed Bloch and Rittenberg believe instead that oxidation at the $\beta$ carbon atom is the first step in the catabolism of propionic acid

The extent to which these deuterium-containing compounds were converted to cholesterol very closely paralleled their ability to acetylate phenyl amino butyric acid (12)

Evidence obtained by the isotopic method has thus given support to the belief that fatty acids are metabolized with the formation of a two-carbon intermediate Other experiments reported by Muñoz and Lelour (96, 80) and by Lehninger (78) indicate that phosphorylation reactions also play a rôle in fatty acid oxidation The components of the enzyme system oxidizing fatty acids in liver extracts have been described by the former investigators The addition of cytochrome c, fumaric acid, inorganic phosphate and adenylic acid to the extract is essential for the optimal oxidation of the fatty acids to ketone bodies

Lehninger (78) has studied the factors involved in the oxidation of octanoic acid by rat liver homogenates and has confirmed the results of Muñoz and Lelour Under the conditions of these experiments, adenosine triphosphate and adenosine diphosphate could be substituted for fumaric acid, the oxidation of which is presumably responsible for the phosphorylation of adenylic acid From these experiments, Lehninger has suggested the intermediate formation of acyl phosphates in octanoic acid oxidation Preliminary experiments indicate that synthetic acyl phosphates having from 8 to 16 carbon atoms do not require adenosine triphosphate for oxidation After hydrolytic destruction by acid, these synthetic preparations do require adenosine triphosphate activation These results coupled with the isotopic experiments of Weinhouse et al indicate that phosphorylation and the formation of a two-carbon intermediate are important stages in the oxidation of fatty acids

c *Complete oxidation of fatty acids and ketone bodies* The reactions concerned with the complete oxidation of fatty acids and ketone bodies to carbon dioxide and water have for many years remained obscure The possibility of the conversion of fatty acids and their derivatives into carbohydrate during their metabolism has undergone critical experimental examination both *in vitro* and *in vivo* Weil-Malherbe (142), for example, has reported that there is an increase of the carbohydrate content of rat liver slices metabolizing acetoacetate Stadie, Zapp and Lukens (124), however, accounted for this newly formed carbohydrate on the basis of the disappearance of carbohydrate precursors preformed in the tissue At present, there are no decisive experiments *in vivo* demonstrating the conversion of the naturally occurring fatty acids to carbohydrate Even such a readily metabolized fatty acid as butyric acid does not give rise to liver glycogen in the normal rat (43, 40, 39, 42) nor to "extra carbohydrate" in the phlorhizinized animal (103) Although the application of classical analytical methods has given little or no support to the theory of conversion of fatty acids to carbohydrates, it has not been possible to deny that fatty acids may be metabolized by way of reactions common to carbohydrate oxidation even though "new carbohydrate" is not formed

The report by Lynen (85) and Virtanen and Sundman (140) that acetate is metabolized by way of the tricarboxylic acid cycle in yeast has been an impetus for further investigation of the metabolism of this compound in mammalian

tissues. Breusch (20, 21) and Wieland and Rosenthal (146) have independently reported the existence of an enzyme system in mammalian tissues which catalyzes the condensation of oxalacetate and acetoacetate or acetate forming citrate Using kidney brei, Wieland and Rosenthal found that when acetate, acetoacetate, or oxalacetate was incubated separately, small amounts of citrate were formed The yield was considerably increased, however, by incubating oxal acetate with either of the other two substances Using muscle extracts, Breusch has obtained essentially the same results Moreover, Breusch has reported that his muscle extracts are not specific for the oxidation of acetoacetate alone, but can oxidize $\beta$ keto derivatives of long chained fatty acids

Krebs and Eggleston (75), however, have recently criticized the work of these investigators Using minced sheep heart, they confirmed the stimulatory effect of dicarboxylic acids on the disappearance of acetoacetate, and the formation of citric acid This effect was greater under anaerobic than under aerobic conditions A quantitative determination of the products of reaction of an anaerobic experiment showed that the extra acetoacetate removed by interaction with oxalacetate could be accounted for as $\beta$ hydroxybutyric acid Krebs and Eggleston have explained their results on the basis of the following equations

$$\left.\begin{array}{l} 3 \text{ oxalacetic acid} \rightarrow \text{malic acid} + \text{tricarboxylic acid} + 2\ CO_2 \\ \text{malic acid} + \text{acetoacetic acid} \rightarrow \text{oxalacetic acid} + \beta \text{ hydroxybutyric acid} \end{array}\right\} (17)$$

According to these authors, acetoacetic acid acts as a hydrogen acceptor converting malic acid into oxalacetic acid and hence makes more of this latter compound available for tricarboxylic acid synthesis Under the conditions of their experiments, oxalacetate did not stimulate acetoacetate disappearance aerobically

The problem of the oxidative conversion of acetate and acetoacetate into the acids of the tricarboxylic acid cycle has also recently been studied using $C^{13}$ (26) Citrate, $\alpha$ ketoglutarate, succinate, fumarate, malate, or oxalacetate, when added in proper concentrations to homogenates of guinea pig kidney cortex, stimulates aerobically the disappearance of acetoacetate Fumaric and $\alpha$ ketoglutaric acids were isolated after adding isotopic acetoacetate together with an excess of some dicarboxylic acid of the tricarboxylic acid cycle A similar study was made of the metabolism of acetate *in vitro* (26) All of the isolated acids contained an excess of $C^{13}$ in an order of magnitude which would be expected if the acids of the tricarboxylic acid cycle are a major pathway of acetate and acetoacetate oxidation These results are in disagreement with the interpretations that Krebs and Eggleston made of their experiments

The fumaric acid isolated after the metabolism of isotopic acetoacetate contained isotopic carbon only in the carboxyl positions The $\alpha$-ketoglutarate contained an excess of $C^{13}$ in both carboxyl groups, but the $C^{13}$ in the carboxyl group $\gamma$ to the keto group was considerably greater than in the other carboxyl position If citric acid were an intermediate in acetoacetate oxidation, as postulated by Breusch and by Wieland and Rosenthal, the two carboxyl carbon atoms of $\alpha$-ketoglutarate should have contained equal concentrations of $C^{13}$

The oxidation of acetoacetate may occur by a mechanism similar to that suggested by Wood et al (154) for pyruvate In this series of reactions (fig 1), cisaconitic acid is the condensation product of oxalacetic acid and a two-carbon compound, either acetic acid or a derivative of acetic acid

The finding that acetic and acetoacetic acids may be metabolized by way of a tricarboxylic acid cycle leads to modification of the present theory of the oxidation of pyruvic acid It has been previously proposed that oxalocitraconic acid is the product of condensation of pyruvic and oxalacetic acids and that this condensation product compound is further oxidized to cisaconitic acid (71) An alternative interpretation is that pyruvate is first oxidatively decarboxylated to a two-carbon intermediate before being condensed with oxalacetate to form cisaconitate By such a series of reactions, pyruvate, acetoacetate and fatty acids would have a common intermediate of metabolism (fig 1) There is objection to regarding acetate as an intermediate of pyruvate oxidation since the oxidative metabolism of pyruvate is considerably greater than acetate in several tissues (71) It has often been suggested that a more metabolically active form of acetate is the product of pyruvate oxidation rather than acetate itself (82, 67, 90) Of these possible active two-carbon intermediates, acetyl phosphate has received greatest attention (82) Knowledge, however, of the reactions of acetyl phosphate in mammalian tissues is so limited that it is impossible to assign it a rôle in oxidative reactions of mammalian tissues at this time

The mechanism of oxidation of short chain fatty acids has been studied *in vivo* with isotopic carbon (25) Acetic, propionic or butyric acid, each labelled with $C^{11}$ in the carboxyl position, was administered to fasted rats together with glucose to insure an adequate formation of glycogen during a two hour experimental period The experiments were conducted on the hypothesis that if any of these fatty acids were oxidized via pathways which involved any of the carbohydrate intermediates or any compounds with which the carbohydrates were in dynamic equilibrium, the isotopic carbon of the fatty acid fed should be found in newly formed glycogen carbon The amount of radioactivity present in the glycogen carbon should be directly proportional to the fraction of the total isotopic material oxidized via the carbohydrate pathway and also directly proportional to the ratio $\frac{\text{amount of glycogen deposited}}{\text{size of effective carbohydrate pool}}$ Thus, the greater the proportion of the carbohydrate pool converted to glycogen, the greater the radioactivity in the glycogen

The glycogen isolated after feeding each of the three fatty acids with glucose contained radioactive carbon Before drawing conclusions regarding the origin of the glycogen carbon, it was necessary to estimate in each case the amount of radioactivity which resulted from the incorporation of the $C^{11}O_2$ produced during the metabolism of the acids All of the radioactivity of the glycogen carbon after acetate feeding could be accounted for by the incorporation of $C^{11}O_2$ derived from the metabolism of the labelled acetate The glycogen isolated after feeding isotopic butyrate or propionate contained an amount of isotopic carbon significantly in excess of that originating from isotopic $CO_2$ These results indi-

cated that two or more carbon atoms of the butyric and propionic acid molecules contributed in part to the glycogen carbon, but the results of the experiments with acetate gave no indication of two-carbon unit incorporation in glycogen

Rittenberg and Bloch (104) have repeated these experiments under somewhat different conditions They included acetate labelled with $C^{13}$ in the carboxyl position in the diet of white rats for a week. The liver glycogen isolated at the end of the experiment contained a small excess of $C^{13}$ which could not be entirely accounted for by carbon dioxide assimilation

The question of the conversion of acetic acid into glycogen has also been studied by Lifson, Lorber and Wood (81) They have isolated liver glycogen from rats two hours after receiving glucose and carboxyl isotopic acetate, and degraded it by chemical and bacteriological methods for the purpose of identifying the position of the isotope in the glucose unit of the glycogen $C^{13}$ was found exclusively in the third and fourth carbon atoms of the glucose unit These positions likewise contained isotope when glycogen was formed from glucose in the presence of isotopic bicarbonate (147) The similarity of the location of $C^{13}$ in the glucose molecule after the administration of carboxyl isotopic acetate and isotopic bicarbonate would have been expected in view of the fact that during the metabolism of both of these compounds, carboxyl isotopic organic acids of the tricarboxylic acid cycle may be formed (fig 2) Lifson and his co-workers are repeating these experiments with acetic acid labelled on both carbon atoms (81) The finding of $C^{13}$ in the glucose molecule other than the third and fourth positions would definitely establish whether or not acetate carbon enters directly as a two-carbon unit into the glycogen molecule The experiments of Lifson et al with carboxyl isotopic acetate have indicated that the reaction

$$CH_3C^{13}OOH + CO_2 + 2H \rightarrow CH_3C^{13}OCOOH + H_2O \qquad (18)$$

does not take place to a significant extent in the rat Glucose units derived from pyruvate formed according to this reaction would have contained $C^{13}$ in the first or second and fifth or sixth carbon atoms (fig 3)

Further evidence in vivo for the conversion of acetic acid into components of the tricarboxylic acid cycle has been supplied by experiments of Rittenberg and Bloch (105) After feeding carboxyl isotopic acetic acid to rats, aspartic and glutamic acids containing isotope in both carboxyl positions have been isolated

It is not necessary to postulate the over all conversion of fatty acids to carbohydrate to explain the appearance of isotopic carbon in glycogen isolated from rats fed isotopic fatty acids and non-isotopic glucose If butyric acid, for example, is oxidized by the series of reactions proposed for acetoacetic acid, oxalacetic acid containing isotopic carboxyl carbon would be formed according to the following reaction

$$CH_3\,CH_2\,CH\,C^{13}OOH + 2O \rightarrow CH_3\,C^{13}OOH + CH_3\,COOH \qquad (19)$$

$$CH_3\,C^{13}OOH + HOOC\,CH_2\,CO\,COOH \xrightarrow[\text{reactions}]{\text{several}} 2\,CO_2$$

$$+ HOOC\,CH_2\,CO\,C^{13}OOH \qquad (20)$$

The oxidation of acetoacetate may occur by a mechanism similar to that suggested by Wood et al (154) for pyruvate In this series of reactions (fig 1), cisaconitic acid is the condensation product of oxalacetic acid and a two-carbon compound, either acetic acid or a derivative of acetic acid

The finding that acetic and acetoacetic acids may be metabolized by way of a tricarboxylic acid cycle leads to modification of the present theory of the oxidation of pyruvic acid It has been previously proposed that oxalocitraconic acid is the product of condensation of pyruvic and oxalacetic acids and that this condensation product compound is further oxidized to cisaconitic acid (71) An alternative interpretation is that pyruvate is first oxidatively decarboxylated to a two-carbon intermediate before being condensed with oxalacetate to form cisaconitate By such a series of reactions, pyruvate, acetoacetate and fatty acids would have a common intermediate of metabolism (fig 1) There is objection to regarding acetate as an intermediate of pyruvate oxidation since the oxidative metabolism of pyruvate is considerably greater than acetate in several tissues (71) It has often been suggested that a more metabolically active form of acetate is the product of pyruvate oxidation rather than acetate itself (82, 67, 90) Of these possible active two-carbon intermediates, acetyl phosphate has received greatest attention (82) Knowledge, however, of the reactions of acetyl phosphate in mammalian tissues is so limited that it is impossible to assign it a rôle in oxidative reactions of mammalian tissues at this time

The mechanism of oxidation of short chain fatty acids has been studied *in vivo* with isotopic carbon (25) Acetic, propionic or butyric acid, each labelled with $C^{11}$ in the carboxyl position, was administered to fasted rats together with glucose to insure an adequate formation of glycogen during a two hour experimental period The experiments were conducted on the hypothesis that if any of these fatty acids were oxidized via pathways which involved any of the carbohydrate intermediates or any compounds with which the carbohydrates were in dynamic equilibrium, the isotopic carbon of the fatty acid fed should be found in newly formed glycogen carbon The amount of radioactivity present in the glycogen carbon should be directly proportional to the fraction of the total isotopic material oxidized via the carbohydrate pathway and also directly proportional to the ratio $\frac{\text{amount of glycogen deposited}}{\text{size of effective carbohydrate pool}}$ Thus, the greater the proportion of the carbohydrate pool converted to glycogen, the greater the radioactivity in the glycogen

The glycogen isolated after feeding each of the three fatty acids with glucose contained radioactive carbon Before drawing conclusions regarding the origin of the glycogen carbon, it was necessary to estimate in each case the amount of radioactivity which resulted from the incorporation of the $C^{11}O_2$ produced during the metabolism of the acids ' All of the radioactivity of the glycogen carbon after acetate feeding could be accounted for by the incorporation of $C^{11}O_2$ derived from the metabolism of the labelled acetate The glycogen isolated after feeding isotopic butyrate or propionate contained an amount of isotopic carbon significantly in excess of that originating from isotopic $CO_2$ These results indi-

studied with isotopes of sulfur and carbon. Tarver and Schmidt (133) using radioactive $S^{35}$ methionine demonstrated that the sulfur of methionine is converted into cystine sulfur. The metabolic origin of the carbon moiety of cystine, however, has been in doubt. In order to ascertain whether methionine contributes its carbon chain to the synthesis of cystine, du Vigneaud and co-workers have synthesized (65) and administered (139) methionine labelled with $S^{34}$ and with $C^{13}$ in the $\beta$ and $\gamma$ positions. Cystine isolated from the hair of these rats contained large amounts of $S^{34}$ but no $C^{13}$, thus demonstrating that the carbon of cystine is not derived from methionine. These results are in accord with previous data obtained by du Vigneaud and co-workers and by Stetten. Binkley and du Vigneaud (8) have reported that liver slices synthesize cysteine in the presence of both homocysteine and serine. Methionine could not be substituted for homocysteine in this isolated system, however. They have postulated that cysteine is formed by the splitting of cystathionine (138, 7, 6), a thio ether, resulting from the interaction of serine and homocysteine. Stetten (126), independently, found that rats fed serine containing $N^{15}$ form cystine with relatively high concentrations of $N^{15}$. Since the $N^{15}$ of the cystine was greater than that of glutamic acid, it could not be attributed to transamination reactions, but rather to the direct utilization of serine in cystine synthesis.

The relatively few experiments so far carried out with amino acids labelled with isotopic carbon illustrate the type of valuable information which can be obtained and are doubtless but forerunners of more extensive studies along similar lines.

## SUMMARY

From the above review one may summarize certain outstanding additions to knowledge which have modified the points of view from which one must approach problems of metabolism.

*a.* $CO_2$ may no longer be considered as simply an end product of metabolism but must be thought of as an essential compound in certain metabolic reactions in higher as well as lower forms of life.

*b.* Instead of considering compounds, such as lactic acid, as specific precursors of more complex compounds, such as glycogen, they must be considered as contributors to the metabolic pool from which the complex compounds are formed.

*c.* More intimate information is now available of the reactions relating carbohydrate, fat and protein precursors and their contribution to the metabolic pool of each.

This is but the beginning. It is fully to be expected that when these tools which permit the tracing of atoms in the body have widespread use, it will be found that many of the earlier conclusions regarding the fate of substances will require drastic revision, and that many problems which hitherto had no means of approach will become vulnerable to attack.

The authors are indebted to many colleagues who have made helpful suggestions during the preparation of this review, and particularly to Prof. D. Wright Wilson, who was good enough to endure and improve several revisions.

(93)*Medes, G , S Weinhouse and N F Floyd Fed Proc 4 98, 1945
(94)*Meyerhof, O , P Ohlmeyer, W Gentner and H Maier-Leibnitz Biochem Ztschr 298 396, 1938
(95) Meyers, V C and M S Fine J Biol Chem 14 9, 1913
(96) Muñoz, J M and L F Leloir J Biol Chem 147 355, 1943
(97) van Niel, C B Advances in enzymology 1 263, 1941
(98) van Niel, C B This Journal 23 338, 1943
(99)*van Niel, C B , J O Thomas, S Ruben and M D Kamen Proc Natl Acad Sci 28 157, 1942
(100)*Nishina, Y , S Endo and H Nakayama Sci Papers, Inst Phys Chem Research, Tokyo 38 341, 1941
(100a) Ochoa, S and E Weisz-Tabori J Biol Chem 159 245, 1945
(100b) Ochoa, S J Biol Chem 159 243, 1945
(101)*Olsen, N S , A Hemingway and A O Nier J Biol Chem 148 611, 1943
(102) Pflüger, E Pflüger's Arch 54 334, 1893
(103) Ringer, A I J Biol Chem 14 43, 1913
(104)*Rittenberg, D and K Bloch J Biol Chem 154 311, 1944
(105)*Rittenberg, D and K Bloch J Biol Chem 157 749, 1945
(106)*Rittenberg, D and R Schoenheimer J Biol Chem 121 248, 1937
(107)*Rittenberg, D and H Waelsch J Biol Chem 136 799, 1940
(108) Rose, W C and F W Dimmitt J Biol Chem 26 345, 1916
(109)*Ruben, S J Am Chem Soc 65 279, 1943
(110)*Ruben, S , W Z Hassid and M D Kamen J Am Chem Soc 61 661, 1939
(111)*Ruben, S and M D Kamen Proc Natl Acad Sci 26 418, 1940
(112)*Ruben, S and M D Kamen J Am Chem Soc 62 3451, 1940
(113)*Ruben, S , M D Kamen and W Z Hassid J Am Chem Soc 62 3443, 1940
(114)*Ruben, S , M D Kamen and L H Perry J Am Chem Soc 62 3450, 1940
(115)*Schoenheimer, R Dynamic state of body constituents Cambridge, 1942
(116)*Schoenheimer, R , S Ratner, D Rittenberg and M Heidelberger J Biol Chem 144 541, 1942
(117)*Schoenheimer, R , S Ratner, D Rittenberg and M Heidelberger J Biol Chem 144 545, 1942
(118)*Schoenheimer, R and D Rittenberg J Biol Chem 120 155, 1937
(119*)Slade, H D and C H Werkman Arch Biochem 2 97, 1943
(120)*Slade, H D , H G Wood, A O Nier, A Hemingway and C H Werkman J Biol Chem 143 133, 1942
(121)*Solomon, A K , B Vennesland, F W Klemperer, J M Buchanan and A. B Hastings J Biol Chem 140 171, 1941
(122) Stadie, W C J Clin Investigation 19 842, 1941
(123) Stadie, W C , J A Zapp, Jr and F D W Lukens J Biol Chem 132 423, 1940
(124) Stadie, W C , J A Zapp, Jr and F D W Lukens J Biol Chem 137 63, 1941
(125) Stare, F J and C A Baumann Proc Roy Soc , B 121 338, 1936
(126)*Stetten, DeW , Jr J Biol Chem 144 501, 1942
(127)*Stetten, DeW , Jr and G E Boxer J Biol Chem 155 231, 1944
(128)*Stetten, DeW , Jr and G E Boxer J Biol Chem 156 271, 1944
(129)*Stetten, DeW , Jr , G E Boxer and B Klein Fed Proc 4 106, 1945
(130)*Stetten, DeW , Jr and R Schoenheimer J Biol Chem 133 329, 1940
(131)*Swendseid, M E , R H Barnes, A Hemingway and A O Nier J Biol Chem 142 47, 1942
(132) Szent-Györgyi, A Ztschr physiol Chem 235 1, 1935, 244 105, 1936
(133)*Tarver, H and C L A Schmidt J Biol Chem 130 67, 1939
(134)*Ussing, H H Skand Arch Physiol 77 85, 1937
(135)*Utter, M F , F Lipmann and C H Werkman J Biol Chem 154 723, 1944, 158 521, 1945

(136)*Vennesland B A K Solomon, J M Buchanan, R D Cramer and A B Hastings J Biol Chem 142: 871, 1942
(137)*Vennesland B, A K Solomon J M Buchanan and A B Hastings J Biol Chem 142: 379, 1942
(138) du Vigneaud, V G B Brown and J P Chandler. J Biol Chem 143: 59, 1942
(139)*du Vigneaud V, G W Kilmer, J R. Rachele and M Cohn J Biol Chem 155: 645, 1944
(140) Virtanen A J and S Sundman Biochem Ztschr 313: 236 1942
(141) Voit, C Cited by O Folin Am J Physiol 13: 117 1905
(142) Weil-Malherbe, H Biochem J 32 2276 1938
(143)*Weinhouse, S, G Medes and N F Floyd J Biol Chem 153: 689 1944 155: 143, 1944
(144)*Weinhouse S, G Medes and N F Floyd J Biol Chem. 158 411, 1945
(145)*Werkman, C H and H. G Wood Advances in enzymology 2 135 New York 1942
(146) Wieland, H and C Rosenthal. Ann Chem 554 241 1945
(147)*Wood, H G, N Lifson and V Lorber J Biol Chem 159 475, 1945
(147a)*Wood, H G, B Vennesland and E A Evans, Jr. J Biol Chem 159 153 1945
(148) Wood, H G and C H Werkman Biochem J 30 48 1936
(149) Wood, H G and C H Werkman Biochem J 32 1262 1938
(150)*Wood H G, C H Werkman A Hemingway and A O Nier J Biol Chem 135 789 1940
(151)*Wood H G, C H Werkman A Hemingway and A O Nier J Biol Chem 139 365 1941
(152)*Wood, H G, C H Werkman A. Hemingway and A O Nier J Biol Chem 139 377, 1941
(153)*Wood, H G, C H Werkman A Hemingway and A. O Nier. Proc Soc Exper Biol and Med 46 313 1941
(154)*Wood H G, C H Werkman, A. Hemingway and A O Nier J Biol Chem 139 483, 1941, 142: 31 1942
(155) Woods, D D Biochem J 30: 515 1936

# YAWNING AND ASSOCIATED PHENOMENA

A PRICE HEUSNER[1]

*Department of Psychiatry, Massachusetts General Hospital and the Department of Neurology and Psychiatry, Harvard Medical School*

In the course of some studies (1) on the vasomotor activity in human fingers I noted that definite circulatory changes occur during spontaneous yawning An attempt to correlate these observations with existing data on yawning revealed that the few pertinent studies of this subject have not found their way into British or American publications Although yawning and its associated phenomena appear to be of minor importance they are not without interest, and it would seem worth while to make readily available the little that is known and the rather more that has been postulated on this subject Such is the purpose of this paper

LITERATURE *Standard sources* It is well to review the popular conceptions yawning as set forth in the standard physiologic, psychologic and lay publica before turning to the special literature

well-known text-books of physiology eight (2, 3, 4, 5, 6, 7, 8, 9) contain on of yawning One of them (10), in a section devoted to "modified ements," defines it in the following terms

> emotional reactions manifest themselves not only by facial expressions, tive changes in respiratory acts Yawning is associated with a pro- during which the mouth is stretched wide open, followed by a short 386)

text-book of Schäfer (11), in a discussion of "modified respiratory " does not go beyond stating

> sists of a deep inspiration accompanied by a wide opening of the mouth and often attended by movements of the arms (Vol II, p 309)

ponderous German hand-books on physiology treat the subject in ory fashion Thus in Hermann's *Handbuch der Physiologie* (12), under the heading "special forms of respiratory movements," yawning is defined and dismissed in these terms

> [yawning is] a psychically or reflexly excited inspiration during which the vocal cords may be set in motion (B IV, H 2, S 234)

In Nagel's *Handbuch der Physiologie des Menschen* (13) the topic is introduced and concluded with the following remarks in a section devoted to "special forms of respiration"

> The yawn is a deep inspiration carried out with widely opened glottis and usually with opened mouth, it is frequently accompanied by movements of the arms, *etc* It is caused by certain psychic influences, fatigue, *etc* (B I, S 27)

[1] Present Address Department of Neurosurgery, Boston City Hospital, Boston, Mass

From the foregoing it is apparent that while none of these authors makes any conjecture as to the physiologic significance of the act, they have all focused their attention upon its respiratory component and have classified it as a respiratory phenomenon. It is hardly necessary to point out that the subject has not been developed beyond or even through the descriptive phase

The psychologic literature appears to be equally barren Yawning has been defined (14) as " a reflex usually induced by bodily fatigue," but critical studies in support of this conception are wanting In a recent review of the psychologic aspects of the subject Moore (15) points out that nothing about yawning has been established beyond the notation that it appears very early in extra uterine life

Articles for lay consumption are more explicit and display less hesitancy in assigning a significance or purpose to the yawn This is evident in the following excerpt from a recent *New York Times Magazine* (16)

Boredom hunger overeating drowsiness and bad ventilation have all been blamed for yawns, but the specific stimulus that starts the reflex is still obscure The power of suggestion has a lot to do with it Yawns are catching as everyone knows and they can even be self induced A yawn has all the beneficial effects of a deep breath of fresh air It is relaxing and refreshing It tones up the muscles of the mouth the chest the back and even the arms Whether it has any other psychological or physical effects science doesn t say

The above views are essentially the same as those which Clarkson (17) submitted to the laity in *Hygeia* They constitute a modern version of the Delsartean concept (18) which we will review in a later paragraph.

It is of interest to compare all of the foregoing descriptions with the dictionary definitions of the word *yawning* and its alleged synonyms, *pandiculation*, *oscitation* and *chasma* Standard lay (19, 20) and medical (21) dictionaries agree that the word *yawning* is derived from the Old English verb *ganien* which has always denoted an "opening" in the sense of "to gape " The definitions of yawning given by the lay dictionaries (19, 20) are in such complete harmony that only one need be quoted

An involuntary act, excited by drowsiness, *etc* and consisting of a deep and long inspiration usually following several successive attempts at inspiration the mouth, fauces, *etc*, typically being wide open (20)

Some medical dictionaries (21) conform to this restricted definition, but others have expanded the meaning to include the associated stretching of the limbs. Thus Dorland (22) offers the following definition of yawning

Pandiculation a deep involuntary inspiration with the open mouth, often accompanied by the act of stretching

From this it also appears that Dorland regards pandiculation and yawning as synonyms

Both lay (19, 20) and medical (21, 22) dictionaries agree that *pandiculation* is derived from the Latin verb *pandiculari*, meaning "to stretch oneself " Of these dictionaries only Dorland, as already noted, offers this word as a synonym

for yawning The others are in agreement on a more narrow definition of which the following is characteristic

A stretching and stiffening, esp of the trunk and extremities, as when fatigued and drowsy, or after waking from sleep (20)

All of these dictionaries (19, 20, 21, 22) offer the words *chasma* (from the Latin noun *chasma* .a cleft or an abyss) and *oscitation* (from the Latin verb *oscitare* to open the mouth wide) as synonyms for the noun *yawn*

From the above it is evident that the authorities, both lay and medical, have been aware of the complexity of the act under consideration and have been uncertain just how much of it to include under the designation "yawn " On semasiological grounds it would appear desirable to preserve a distinction between yawning and pandiculation, and to employ some such expression as "the yawn-stretch act" when the entire complex is intended

*Special sources* (Occurrence) Depending on the breadth of definition adopted, the occurrence of yawning throughout the animal kingdom is more or less widespread Crämer (23) points out that the familiar gaping movements of fishes, amphibians and reptiles simulate the oral component of yawning in man, but most observers (24, 25, 26, 27) decline to read true yawning into this superficial resemblance Whether or not the gaping of these lower forms is accompanied by the respiratory component of yawning and a generalized stretching is not known, and it is best to follow Peiper (27) in reserving judgment on the occurrence of yawning in these classes

The data on birds are little more complete, although the gaping chicken beating its wings on tip-toe is an everyday spectacle Here a generalized stretching is seen in conjunction with gaping, but the question of an associated respiratory effort has not been answered by critical observation It is the impression of both Heinroth (25) and Crämer (23) that true yawning is practiced by birds, but Hauptmann (26) opposes this view

It is generally agreed (23, 24, 25, 26, 27) that among terrestrial mammals true yawning and stretching are to be observed in primates and carnivores The situation with regard to herbivora is not so clear, although most authors concede that these animals practice stretching The failure of the herbivora to exhibit the gaping component of the yawn-stretch act constitutes an objection standing in the way of admitting them into the category of yawners, but Dumpert (24) has pointed out that since herbivora are exclusively nose-breathers, even under conditions of stress, it is unnecessary for them to add the oral component to the yawn-stretch act in order to effect its inspiratory phase

Before leaving the subject of yawning in animals it is important to mention the possible relationship of its gaping component to the "automatic expressions" described by Darwin (28) The gaping movements of fishes, amphibians and reptiles may belong to this group of emotional expressions, and it appears certain that some of the anthropoids employ the mouth-act in question for emotional attitudinization Finally, as we shall see, there are those who regard the complete yawn-stretch complex as but another example of the automatic expressions like laughing and crying

In the case of man little can be added to the previously noted lay observations on occurrence The idea that yawns are "catching" can be traced back to Mayer (29) who believes that in some individuals the threshold for this so-called psychically released yawning is exceptionally low, and that in such persons autosuggestion may constitute an effective stimulus Crämer (23) and Schrijver (30) hold that yawning may normally be a symptom of hunger They report that it is so regarded by some of the Continental laity, and Luciani (31) has been cited as supporting this view It should be noted, however, that yawning was not among the symptoms of the fasting men observed by Carlson (32)

It has been remarked (24, 33) that among infants and children, as among many animals, yawning is more regularly attended by stretching than is the case among adults Dumpert (24) looks upon the adult's yawn, without pandiculation, as a fractionation of a primitive yawn-stretch reaction, and he postulates that such a dissociation is the outcome of acquired voluntary inhibitions cultivated in accordance with the demands of "good company" As we shall see in a subsequent paragraph, this concept enjoys some support from the fact that man's ability to yawn without stretching can be lost in certain disorders of the central nervous system There are other observations, however, which suggest that yawning and stretching are independent albeit frequently associated acts, rather than fundamentally conjoined responses In the first place, infants under 1 year frequently yawn without stretching and it is hardly permissible to regard this as a fractionation of some basic physiologic pattern by voluntary inhibitions Secondly, regardless of man's age he displays a tendency to yawn (without stretching) just prior to surrendering to sleep, and to stretch (without yawning) upon waking This too intimates that factors other than voluntary inhibitions are active in determining whether pandiculation and yawning occur together or separately

Yawning under circumstances other than those already mentioned is probably always morbid

*Special sources* (Clinical) Addressing clinicians in 1908 Geigel (34) lamented the fact that his teachers had never mentioned yawning and complained that nowhere could he discover observations elucidating its physiologic or pathologic significance Similar conditions obtain today although morbid yawning is more common than our neglect of it would imply

Geigel's contribution to the subject was quite limited He emphasized the value of yawning as a favorable prognostic sign in certain acute illnesses and noted its occurrence in hysterics. Several observers (24, 27, 35, 36) have remarked the frequency of yawning after severe hemorrhage Peiper (27) seeks to explain this on the basis of his theory (*vide infra*) of lowered excitability of the central nervous system, whereas Nash (36) attributes it to anoxia and/or arterial hypotension There are no experimental data supporting either explanation

Crämer (23) and Schrijver (30) have reported the frequency of excessive yawning in diseases of the gastro-intestinal tract, especially that of duodenal ulcer, wherein crises of yawning sometimes parallel exacerbations of symptoms The association of deranged stools and flatus with morbid yawning so impressed

Crämer that he postulated an auto-intoxication by bacterial toxins arising from within the bowel as the causative agent He reviews the rather meagre evidence supporting this view

Neurologists have met with abnormal yawning rather more frequently than others Oppenheim (37), Geigel (34) and Lewy (35) have encountered it in hysteria, and its occurrence as an aura in epilepsy has been remarked by Oppenheim (37), Lewy (35), Wilson (38) and Penfield (39) In Wilson's experience this aura has been associated with his so-called "peri-ventricular" or "visceral" epilepsy wherein the yawn is followed by voiding of urine, giddiness, headache and sometimes vomiting He suggests that such cases depend on disorder of visceral centers in the third ventricle regulating sleep and fluid interchange, with subsequent spread to others on the floor of the fourth ventricle Penfield (p 110) gives the clinical details of such a case

Among the manifold sequelae of epidemic encephalitis is that of paroxysmal yawning usually in conjunction with other, though varied, neurologic disturbances Mayer (29), Wilson (38) and Sicard and Paraf (40) describe cases from their personal experience, but it has not been possible to ascribe the symptom to proven lesions at particular foci in the neuro-axis In this connection it is of interest to record certain impressions which Yakovlev (41) has formed with regard to derangements of yawning and pandiculation in other disorders involving the basal ganglia In his experience Huntington's Choreics exhibit a propensity for yawning and more especially for stretching which stands in sharp contrast to the behaviour of Parkinsonics who rarely yawn and never stretch Yakovlev emphasizes that these impressions have been derived from a study of relatively few cases and stand in need of confirmation by a test on more material

Excessive yawning is frequently seen in patients harboring expanding intracranial lesions, notably cerebellar abscess or tumor (35, 36, 37, 38, 42) When attended by abnormal drowsiness the sign is without localizing value (37, 38), but its occurrence in an alert patient suggests to some (38) that a thorough search be made for third ventricular and hypothalamic signs Nash (36) hazards an explanation for the yawning seen in the usual run of brain tumor cases He states

> The occurrence of yawning, which is essentially an atypical respiratory act, in cases of brain tumor can be explained by irritation of the brain resulting in "afferent" impulses to the medullary respiratory center, which modify its rhythmic activity (p 92)

*Special sources* (Investigative) Mayer (29) alone has attempted a systematic investigation of the yawn-stretch act By means of the roentgen-screen, laryngeal mirror and palpation of muscles he has analyzed the sequence of events in man when the complete complex, unmodified by inhibition, is allowed to develop For convenience of description he divides the act into a number of phases

I Inspiratory Phase (duration 4 4 to 6 8 sec )
  A Initial Part
  B Acme (duration 2 5 sec )
II Expiratory Phase

During the initial part of the inspiratory phase one observes a widening of the chest, a descent of the diaphragm and larynx, an elevation of the *ala nasi* and soft palate, a downward and backward displacement of the tongue, an abduction of the vocal cords and an opening of the mouth This is occasionally attended by a subjective crackling in the ears which is thought (43) to be due to a contraction of the *M tensor veli palatini* with jerking of its tendon in the bursa between it and the pterygoid hamulus

When the acme of the complex has been attained one notes that the chest diaphragm, larynx, tongue, palate and mouth have maintained or accentuated their respective movements of the initial phase There has, in addition, been a firm closure of the eyes and a rapid development of the stretch component The number and distribution of muscles recruited for the stretch are subject to variation and may be modified by voluntary inhibition Neck and arm muscles are most frequently recruited, but those of the legs, trunk and abdomen may participate The probable influence of such widespread muscular activity upon the venous return, and secondarily upon cardiac filling, rate and output, has been recognized (24, 29), but no studies have been made to confirm this At the close of this phase saliva occasionally spurts from the submaxillary and sublingual ducts, presumably due to a squeezing of these glands by the contracting oral musculature

During the expiratory phase one notes a short sharp expiration with gradual relaxation of all previously activated muscles permitting the return of diaphragm, larynx, tongue, *etc* to their resting positions Lacrimation and swallowing may occur at this stage The former is believed (44) to be due to an augmentation of secretion rather than to a simple compression of the lacrymal glands because the tightest possible voluntary blepharospasm cannot squeeze forth tears in an amount sufficient to overflow the conjunctival sac The swallowing is presumed to depend upon the occurrence of the previously noted salivation This final phase of the yawn is usually accompanied by some such subjective feeling as relief, satisfaction or refreshment

Prior to Mayer's (29) fluoroscopic observations, which show the diaphragm to descend and remain relatively fixed in this position from the initiation until the expiratory phase of the yawn, Hauptmann (20) had arrived at a different opinion as to the behaviour of this organ Using a kymographic method for recording thoracic and abdominal respiration Hauptmann was led to believe that the rhythmic action of this muscle was but little, if at all, interrupted by yawning His view must, of course, give way to that suggested by the more refined method of fluoroscopy It is important to note, however, that Mayer and Hauptmann agree that yawning effects no ultimate increase in aeration of the lungs, they argue that the apneic period following the yawn (see fig 1 and 2) more than compensates for any temporary increase in aeration afforded by a single deep breath

The neural structures subserving the complicated yawn stretch act may conveniently be thought of as comprising an integrative center played upon by diverse afferent elements and discharging over its several efferent channels Evi

dence at hand is insufficient, however, to establish the location or even the existence of a "yawn-center" in man or animals Ellenberger and Scheunert (45) include yawning among the functions of the medulla oblongata in dogs, but they do not submit the evidence supporting this localization In man a postulated center has been variously located in the *corpus striatum* (23, 35) and "subcortical ganglia" (24, 27, 29) on very unsatisfactory evidence There are, however, two significant papers bearing on this question Gamper (46) has seen the complete yawn-stretch act in what he describes as a mid-brain preparation (*Arhinencephalie mit Encephalocele*), and Catel and Krauspe (47) have observed it in a case where only the medulla oblongata and more caudal portions of the nervous system were

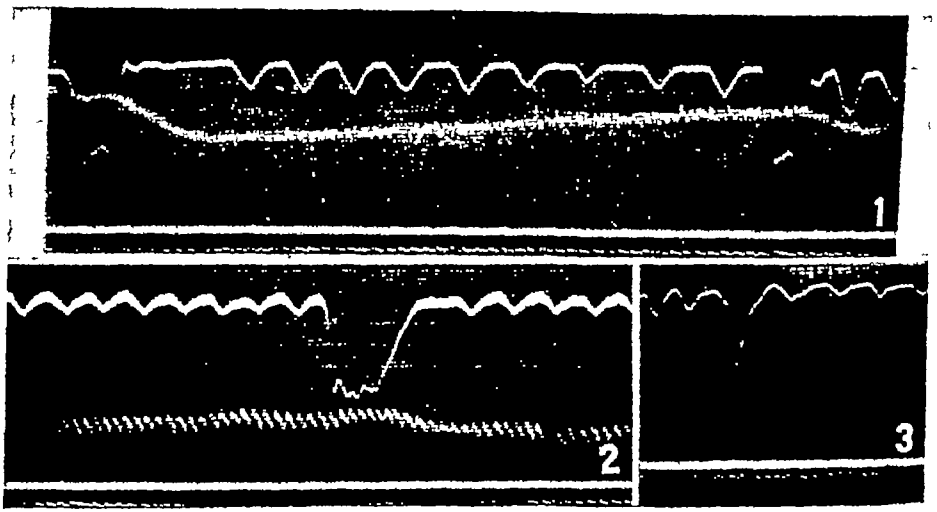



Fig 1 Record of vasoconstriction in normal finger accompanying yawning
Fig 2 Record of vasoconstriction in sympathectomized finger accompanying yawning
Fig 3 Record of vasoconstriction in normal finger accompanying deep breath The tracings from above downward are (a) respiration by stethograph, (b) digital translucency, (c) signal magnet and (d) time in one second intervals

completely formed (*Meroanenzephalie mit Meroankranie*) These observations suggest that if a yawn-center exists it is to be found in the medulla oblongata, possibly in the immediate vicinity of the respiratory and vasomotor centers

The so-called psychically released yawning, or yawning as an apparent consequence of suggestion, implies that the act can be initiated by influences emanating from the highest cerebral levels Inhibition of the act by influences arising at these levels is confirmed by the behaviour of hemiplegic patients who have lost the ability to fractionate the yawn-stretch act Both Dumpert (24) and Lewy (35) have noted yawning in such patients attended by stretching movements confined to the affected (*i e*, "paralyzed") extremities doubtless to the amazement of the patient if not the doctor Dumpert emphasizes that although this phenomenon is not to be seen in all hemiplegics it is consistently present in given cases

Activation and possibly inhibitory modification of yawning by neural elements below the telencephalon is of course attested to by the observations of Gamper (46) and of Catel and Krauspe (47) on their cases of cerebral malformation The involuntary nature of most yawns and their occasional occurrence in coma tose patients (29) likewise argue for activation from so-called "sub-conscious levels," but nothing further is known about the afferent influences exciting this act

Information regarding the centrally located efferent pathways subserving the yawn-stretch act is limited to deductions suggested by the involuntary activation of paralytic limbs during yawning This stretching of limbs deprived of cortico-spinal control appears to indicate that the so-called "extra pyramidal" pathways play a significant rôle in the uninhibited act

*Special sources* (Theories) Numerous hypotheses concerning the mecha nism and significance of yawning and stretching have appeared, and existing data do not suffice to show which of these are tenable They are not mutually ex clusive and it is possible that each contains a grain of truth

The first theory to find its way into print, that of the Delsarteans, appeared in Russell's monograph (18) of 1891 This little book, which remains the most exhaustive account of yawning in the English language, defies description and should be consulted in the original by anyone interested in whimsical physiology Suffice it here to say that yawning is presented as an "automatic impulse" caused by 'bad air in the lungs," designed by Nature as a 'gymnastic," and intended both to "awaken the respiratory organs into activity" and to effect a stimulation of the brain through increased activity of the circulation " Although this con cept enjoys no supporting evidence it appears to be the fore runner of present day lay belief (16, 17) and it is not without current favor among clinicians Within the past decade Otto (33) and Seeligmüller (48) have reiterated the notion that yawning is a gymnastic the practice of which should be cultivated as a means of toning up the body or relaxing mental tension

Hauptmann (26) appears to have been the first clinician to attempt an inter pretation of the yawn-stretch act Writing in 1920, he suggested that this complex is a means of combating the loss of muscle tone which, along with tedium, develops as a consequence of enforced inactivity of the higher cerebral centers He emphasized that this augmentation of muscle tone and metabolism, rather than the deep inspiratory effort, is the main purpose of the yawn-stretch act We have already noted his contention that any temporary improvement in aera tion due to this inspiratory effort is more than offset by the period of apnea which follows the expiratory phase By this process of reasoning but without recourse to precise measurements, Hauptmann rejects the notion that yawning bespeaks an anoxia of cerebral tissue This conclusion has been widely accepted (24, 27, 29, 35) without confirmatory evidence

Mayer (29) concluded that the yawn-stretch act is an automatic expression of cerebral fatigue in the sense that crying and laughing are expressions of sorrow and happiness In this conclusion he has been joined by several subsequent writers (10, 27, 35) Mayer goes on to suggest that the act accomplishes im

portant but as yet undefined physiologic adjustments in the state of the circulation and in the metabolism of the recruited muscles

Of all hypotheses advanced that of Dumpert (24) is by far the most elaborate The salient features of his concept are as follows

1 Yawning (the mouth act attended by a deep inspiration) is but part of the larger yawn-stretch complex which occurs in many animals, in infants and in children This larger reaction is first regularly dissociated into its respiratory and stretching components in adult man, and even here the ability to fractionate the act can disappear following certain lesions of the central nervous system
2 The yawn-stretch act is believed to occur whenever the cerebral circulation is not favorably adjusted for maintenance of the waking and alert states
3 The yawn-stretch complex or even yawning alone is thought to effect an augmentation of the venous return to the heart by means of
   a The "milking action" of the striated muscles upon the veins of the limbs,
   b The compressing action of the contracting abdominal muscles and the descending diaphragm upon the veins of the splanchnic bed, and,
   c The aspirating effect of the lowered intra-thoracic pressure upon blood entering the *venae cavae* during the inspiratory phase of the act
4 This presumed increase in venous return is thought to effect an increase in the rates of cardiac filling and output (Bainbridge's reflex), thereby providing a generalized improvement in the supply of arterial blood to the tissues
5 A differential distribution of the supposedly augmented arterial flow is presumed to occur for the especial benefit of the cerebral circulation This selective redistribution is believed traceable to the effects of centripetal impulses arising in the stretched muscles and serving to
   a Produce a widespread systemic vasoconstriction by their action on the vasomotor center (a la Bayliss), and,
   b Effect a cerebral vasodilatation by their action on Weber's center
6 Thus the yawn-stretch reaction and, less effectively, the yawn alone are able to combat cerebral circulatory states unfavorable to the maintenance of the waking and alert conditions

Although much that Dumpert postulates is probable and my own observations (*vide infra*) confirm the occurrence of the systemic vasoconstriction he predicted, his theory is without support at its crucial points Thus it is unjustifiable to assume that the cerebral circulation is diminished during sleep, indeed, the only reliable information (49) we have on the state of the cerebral circulation prior to, during and after sleep fails to disclose any significant variations Further, there are no observations confirming an increase of the venous return during yawning Finally, Dumpert invokes the aid of a cerebro-dilatory center the existence of which is open to question

Peiper (27) looks upon yawning as a purely respiratory act which represents a transient disorder of the respiratory center He believes that as a result of fatigue the excitability or sensitivity (*erregbarkeit*) of the higher respiratory centers (50) is depressed whereupon a lower lying yawn-center is released He thus explains the occurrence of an automatic expression of fatigue and does not postulate that it accomplishes any physiologic adjustments His theory is attractive but rests upon purely hypothetical considerations

One further theory concerning the yawn has come to my attention It has been suggested that as the nuchal muscles are recruited in the stretch they squeeze

upon the thyroid gland, express thyroxine into the blood stream and thereby accelerate the metabolism The literature has been searched in vain for the origin and subsequent reference to this idea

NEW OBSERVATIONS *Methods and material* My observations on yawning were made while studying the vasomotor activity in the digits of adult humans some of whom were "normal" and others of whom were victims of either Raynaud's or (early) Buerger's diseases The diseased group was studied both before and after therapeutic pre-ganglionic sympathectomy None was aware of my interest in yawning

The subject, comfortably seated, placed one finger or toe, without restraining device, on a simple rest between a photo-electric cell and a constant light source Current set up in the cell by light passing through the digit was led to a 2 stage amplifier, whence it emerged to activate a DuBois oscillograph A mirror mounted on the latter directed a beam of reflected light onto a moving strip of bromide paper The electrical circuit employed was such that a downward deflection on the photographic record was produced by an increase in the amount of light (and decrease in the amount of blood) traversing the digit

A stethograph of the air transmission type was applied to the chest and its indicator made to cast a second shadow on the sensitized paper A downward excursion of this indicator was produced by an inspiratory movement

Tracings of a time marker and a signal magnet also appear on the photographic records

Subjects were routinely observed over periods of 30 to 60 minutes in a warm darkened room Under these conditions spontaneous yawning was frequent

*Observations* In the digits of normal adults a vasoconstriction was regularly observed to follow a spontaneous yawn Figure 1 is a characteristic record of the development and subsidence of this phenomenon in a finger From this tracing it can be seen that the vasoconstriction begins to appear after a latency of about 4 to 4.5 seconds as measured from the inception of the inspiratory phase of the yawn The response is maximal within 9 to 10 seconds after the onset of the yawn and then gradually subsides during the ensuing 45 seconds In a large number of such observations the latent period was regularly found to be between 3.5 and 5 seconds The magnitude of the response and the time required for its complete development and subsidence were subject to considerable variation even in the same individual In general these values were roughly proportional to the depth and duration of inspiratory movement Maximal response was nearly always evident within 7 to 12 seconds and subsidence was usually complete within 30 to 60 seconds

It was also observed that yawning in normal adults is regularly attended by a transient cardiac acceleration This speeding of the heart was found to develop and subside *pari passu* with the vasoconstrictor response The subject in figure 1, whose resting pulse is 80/min , shows an acceleration to 90/min This degree of cardiac acceleration is fairly representative of many similar observations but it is subject to variation among different individuals and even in the same individual according to the depth of the yawn.

That the observed changes in the translucency of digits during a yawn are truly vasoconstrictor responses dependent upon the integrity of the autonomic innervation of the part is clearly shown by studies on sympathectomized extremities Prior to surgical intervention the digits of such limbs exhibited responses equal to or even greater than those observed in normal parts Following sympathectomy they show a greatly diminished or no vasomotor response depending upon the completeness of the denervation Figure 2, a tracing from the finger of a Raynaud patient after sympathectomy, shows no appreciable vasoconstriction following a deep yawn It is interesting to note, however, that the cardiac acceleration is not abolished This subject's resting pulse rate of 70/min is raised to one of 80 to 85/min during the inspiratory phase of the yawn

Results in every way comparable to those depicted for fingers were obtained from the toes of normal and sympathectomized subjects

Discussion Although Dumpert (24) and Mayer (29) predicted that circulatory alterations would be found to accompany yawning, there has been, to my knowledge, no previous demonstration of such changes The phenomena under consideration are, however, closely related to or identical with the circulatory changes which Bolton *et al* (51) describe as following a deep inspiration

In a series of experiments on man Bolton *et al* showed that following a deep voluntary inspiration a vasoconstriction occurs in the digital vessels Its reflex nature was conclusively established by experiments on denervated and sympathectomized limbs, and some evidence was adduced in favor of the afferent stimulus arising from the expansion of the chest wall Figure 3 is a recording by our apparatus of the vasomotor reflex they describe

In the discussion of their observations Bolton *et al* make no mention of their reflex being accompanied by a cardiac acceleration Careful scrutiny of their figures 2 and 3 suggests, however, that this did occur in their experiments The subject of my figure 3 exhibited a resting pulse rate of 70/min This rose to 80/min during the 10 seconds following the initiation of the deep inspiration and is typical of the behaviour of the pulse in many such observations

## SUMMARY

The literature on yawning and stretching has been reviewed The descriptive phase of the subject is essentially complete for man The experimental phase is barely begun with the demonstration that circulatory changes consisting of transient cardiac acceleration and digital vasoconstriction attend spontaneous yawning in man Aspects of the problem remaining completely unexplored include

a The behaviour of the cerebral circulation,
b Measurements of cardiac filling and output,
c The chemistry of the respiratory component,
d The nature of the motor discharge in the stretch component, and,
e The possibility of endocrine (*e g* , thyroid) changes

Until such observations are completed, any formulation of the physiological significance of this act will rest upon an insecure foundation

## REFERENCES


(1) Finesinger J E A P Heusner and R H Smithwick Vasomotor studies in Raynaud s disease before and after sympathectomy Tr Am Neurol A 65 193, 1939
(2) Bayliss W M Principles of general physiology 2nd ed Revised Longmans, Green & Co , London, 1918
(3) Lehrbuch der Allgemeinen Physiologie Ed. by E Gellhorn Thieme Leipzig 1931
(4) Wright, S Applied physiology 5th ed , Oxford Univ Press New York 1934
(5) Winton, F R and L E Bayliss Human physiology 2nd ed Blakiston s Son & Co , Inc , Philadelphia, 1937
(6) Macleod s Physiology in modern medicine Ed by P Bard 8th ed Mosby Co , St Louis 1938
(7) Howell, W. H A textbook of physiology 14th ed Saunders Co , Philadelphia 1940
(8) Starling s principles of human physiology Ed by C L Evans 8th ed , Lea & Febiger, Philadelphia, 1941
(9) Best C H and N B Taylor The physiological basis of medical practice 3rd ed , Williams & Wilkins, Baltimore 1943
(10) Wiggers, C J Physiology in health and disease 3rd ed , Lea & Febiger, Philadelphia 1939 p 386
(11) Starling, E H Text book of physiology Ed by E A Schäfer Pentland, Edinburg & London 1900, Vol ii, p 309
(12) Rosenthal, J Handbuch der Physiologie Ed by L. Hermann Vogel Leipzig 1880, B IV, H 2 S 234
(13) Boruttau, H Handbuch der Physiologie des Menschen Ed by W Nagel Vieweg u Sohn Braunschweig 1905, B I S 27
(14) Dictionary of psychology Ed by H C Warren Houghton Mifflin Co New York 1934
(15) Moore, J E Some psychological aspects of yawning J Gen Psychol 27: 289 1942
(16) New York Times Magazine October 29, 1944
(17) Clarkson H E , Jr When the yawn breaks Hygeia 20 585 1942
(18) Russell, H Yawning The Delsarte Series No 1 U S Book Company, New York, 1891
(19) Shorter Oxford English dictionary 2nd ed Clarendon Press Oxford, 1939
(20) Webster's New International Dictionary 2nd ed Unabridged G & C Merriam Co , Springfield, 1942
(21) Gould's Medical Dictionary 4th ed by G M Gould Blakiston, Philadelphia, 1935
(22) The American Illustrated Medical Dictionary 20th ed by W A N Dorland, Saunders Co Philadelphia, 1944
(23) Crämer, F Über Sodbrennen und Gähnen Arch f Verdauungskr 33 149, 1924
(24) Dumpert, V Zur Kenntnis des Wesens und der physiologischen Bedeutung des Gähnens J f Psychol u Neurol 27: 82 1921
(25) Heinroth Quoted by Dumpert (24)
(26) Hauptmann A Wie wann und wozu gähnen wir? Neurol Zentralbl 39 781, 1920
(27) Peiper, A Das Gähnen Deutsch med Wchnschr 58: 693 1932
(28) Darwin, C The expression of the emotions in man and animals Appleton, New York, 1873
(29) Mayer, C Physiologisches und Pathologisches über das Gähnen. Ztschr f Biol 73 101, 1921
(30) Schreiver, J Über das Magengähnen Arch f Verdauungskr 43 378 1928
(31) Luciani Physiologie des Menschen T 1 Jena, 1911 quoted by Schreiver (30)
(32) Carlson A. J Personal communication to Schreiver (30)
(33) Otto H Vom Recken, Strecken, Gähnen und Husten Fortschr d Med 53: 304 1935

(34) GEIGEL, R Das Gähnen Muenchen med Wchnschr **55** 223, 1908
(35) LEWY, E Über das Gähnen Ztschr f Neurol u Psychiat **72** 161, 1921
(36) NASH, J Surgical physiology Thomas, Springfield, 1942, pp 92 and 115
(37) OPPENHEIM, H Lehrbuch der Nervenkrankheiten 5th ed , Karger, Berlin, 1908, pp 1219 and 1445
(38) WILSON, S A K Text book of neurology Ed by A N Bruce, Williams & Wilkins, Baltimore, 1940, Vol I, p 121, and Vol II, pp 1223 and 1507
(39) PENFIELD, W AND T C ERICKSON Epilepsy and cerebral localization Thomas, Springfield, 1941, p 110
(40) SICARD, J A ET J PARAF Fou Rire Syncopal et Baillments au Cours de L'Encephalite Epidémique Bull et mem de soc méd d hôp de Paris **45** 232, 1921
(41) YAKOVLEV, P I Personal communication
(42) MACEWEN, W Pyogenic infective diseases of the brain and spinal cord Maclehose & Sons, Glasgow, 1893, p 195
(43) VALENTIN Ztschr f Ohrenheilk **46** 84, quoted by MAYER (29)
(44) RÖMER Lehrbuch der Augenheilkunde 3 aufl , 1919, p 241, quoted by MAYER (29)
(45) ELLENBERGER, W UND A SCHEUNERT Lehrbuch der vergleichenden Physiologie der Haussäugetiere Parey, Berlin, 1910, p 578
(46) GAMPER, E Bau und Leistungen eines menschlichen Mittelhirnwesens (Arhinencephalie mit Encephalocele) zugleich ein Beitrag zur Teratologie und Faser systematik Ztschr f d ges Neurol u Psychiat **104** 49, 1926
(47) CATEL, W UND C A KRAUSPE Über die nervöse Leistung und den anatomischen Bau einer menschlichen Hirnmissbildung (Meroanenzephalie mit Meroakranie) Jahrb f Kinderh **129** 1, 1930
(48) SEELIGMÜLLER, A Von Gähnen und Räkeln Therap d Gegenw **81** 440, 1940
(49) GIBBS, F A , E L GIBBS AND W G LENNOX The cerebral blood flow during sleep in man Brain **58** 44, 1935
(50) LUMSDEN, T Observations on the respiratory centres in the cat J Physiol **57** 153, 1923
(51) BOLTON, B , E A CARMICHAEL AND G STÜRUP Vaso-constriction following deep

PHYSIOLOGICAL REVIEWS
VOL 26 APRIL, 1946 No 2

# SYMPATHOMIMETIC AMINES THE RELATION OF STRUCTURE TO THEIR ACTION AND INACTIVATION

KARL H BEYER

*From the Department of Pharmacology The Medical Research Division Sharp and Dohme Inc Glenolden Pa*

We are dealing with a range of compounds which simulate the effects of sympathetic nerves not only with varying intensity but with varying precision In some cases the points of chief interest in the action of a compound are those in which it differs from the action of adrenine A term at once wider and more descriptive than adrenine like seems needed to indicate the type of action common to these bases We propose to call it 'sympathomimetic,' a term which indicates the relation of action to innervation by the sympathetic system, without involving any theoretical preconception as to the meaning of that relation or the precise mechanism of the action Barger and Dale J Physiol 41 19 1910

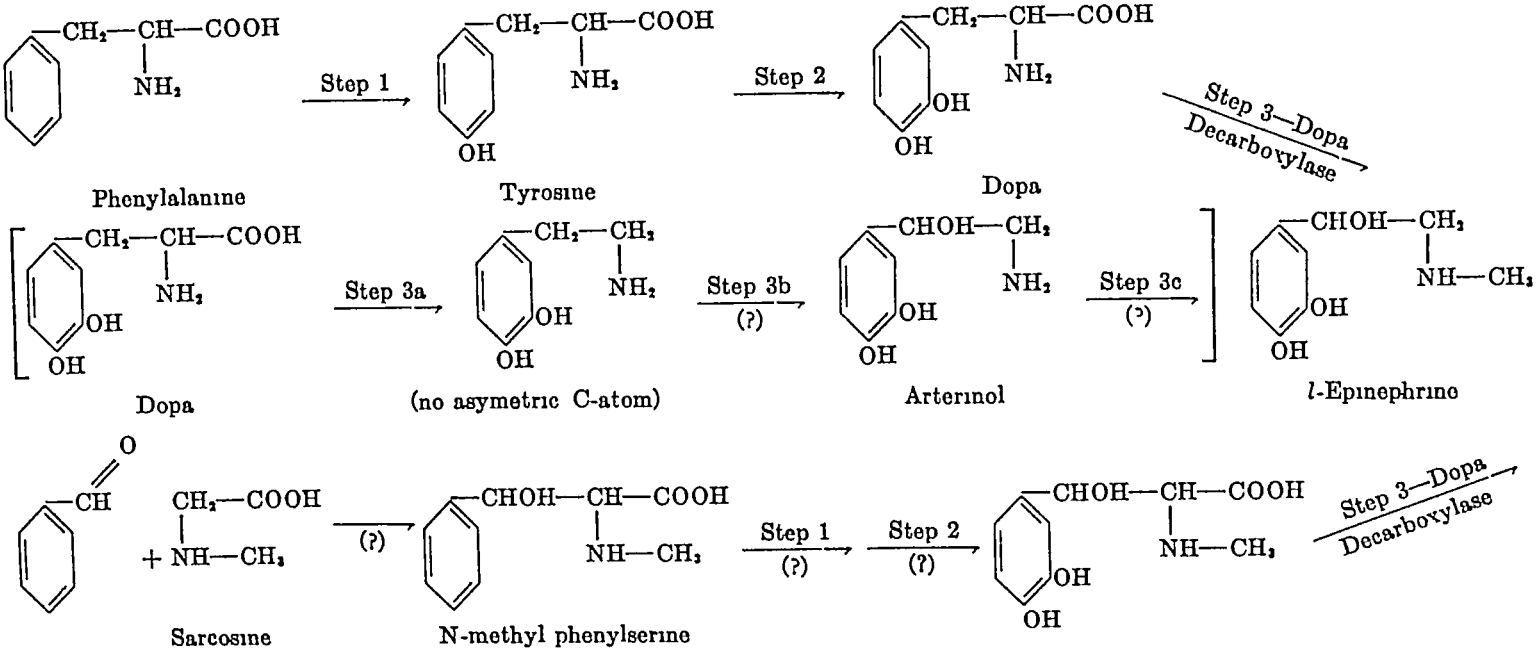
Reviews of the literature on sympathomimetic amines have appeared from time to time Of these the ones by Chen and Schmidt (1) and Hartung (2) are comprehensive integrations of the work in this field through 1931 Since then there has appeared a number of briefer reviews (3), the most recent comprehensive treatment of the subject being the Symposium on Sympathomimetic Agents presented before the American Chemical Society in 1944 (4)

The purpose of this article is to review the formation of pressor amines in the body, and their relation to the pathogenesis and experimental alleviation of hypertension, to consider their pharmacodynamic effects on cellular or tissue metabolism as determining the mode of action of these agents, to review the relation of structure to the inactivation of sympathomimetic amines, and to present certain generalizations concerning the relation of structure of these compounds to their action and inactivation

EPINEPHRINE FORMATION The formation of epinephrine and other pressor amines in the body can be portrayed simply with pencil and paper although proof of the mode of synthesis of the hormone by mammalian tissues is quite incomplete The equations used to illustrate the discussion of epinephrine synthesis are given in the accompanying diagram

In our present discussion of these syntheses it is convenient to begin with phenylalanine since it has been shown to be an essential amino acid (5) Decarboxylation of this amino acid has been demonstrated to occur in the course of bacterial proteolysis with the formation of the pressor agent $\beta$ phenylethylamine (6) This reaction occurs in the digestive tract, the amine being absorbed and normally inactivated by the amine oxidase of the body (7)

The oxidation of phenylalanine to tyrosine (step 1) was shown to occur in the presence of iron and hydrogen peroxide (8) Recently the biological conversion of phenylalanine to tyrosine has received strong substantiation by the isolation of tyrosine containing deuterium from the bodies of rats whose diet has been supplemented with deuterophenylalanine (9) The decarboxylation of tyrosine


Phenylalanine
Step 1
Tyrosine
Step 2
Dopa
Step 3—Dopa Decarboxylase
Dopa
Step 3a
(no asymetric C-atom)
Step 3b
(?)
Arterinol
Step 3c
(?)
l-Epinephrine
Sarcosine
(?)
N-methyl phenylserine
Step 1
(?)
Step 2
(?)
Step 3—Dopa Decarboxylase

to yield the pressor agent tyramine occurs in the kidney (10) and pancreas (11) and is catalyzed by a decarboxylase (10b, 12) Bacteria have been shown to possess a similar carboxylase and very likely the decarboxylation of tyrosine in the intestine contributes considerably to that normally eliminated by the body (13)

The oxidation of tyrosine to 3,4 dihydroxyphenylalanine, "dopa," (step 2) can be initiated by enzymatic and nonenzymatic oxidative measures (14) The ascorbic-dehydroascorbic acid system is capable of oxidizing tyramine to the corresponding 3,4 dihydric derivative and stabilizing the reaction at that point (15) and presumably could do so for tyrosine An analogous point is the stabilization of epinephrine by ascorbic acid in the adrenal gland (16) Also, Medes (17) reported the recovery of *l*-3,4 dihydroxyphenylalanine from urine when large amounts of tyrosine were administered to a patient diagnosed as having tyrosinosis

The method for the decarboxylation of "dopa" in the mammalian organism rests on firmer structure Holtz has demonstrated the presence of "dopa" decarboxylase in practically all animals capable of synthesizing epinephrine (18), and has shown it to function selectively in the decarboxylation of the *l*-amino acid, 3,4 dihydroxyphenylalanine (step 3) He proposed the theory that "dopa" decarboxylase may be an accessory enzyme important for the biological formation of epinephrine His later diagrams show the decarboxylation of "dopa" to 3,4 dihydroxyphenylethylamine by the decarboxylase and another arrow pointing from that structure to one for epinephrine According to the scheme of things as developed to this point there must be introduced into this agent, 3,4 dihydroxyphenylethylamine, both an aliphatic hydroxyl group and a methyl group on the amino nitrogen (steps 3b and 3c) Moreover, this feat must be accomplished under such stable conditions as not to wreck the very labile catechol nucleus Heard and Raper have reported that by the action of tyrosinase on N methyl "dopa" *in vitro* adrenalone was formed However, perfusion of adrenalone through the adrenal gland did not result in its reduction to epinephrine (14b) Also, Vinet claimed that the adrenal medulla was capable of bringing about the oxidation and methylation of decarboxylated dihydroxyphenylalanine (19) An appreciation of the chemistry involved in such a transformation would make it seem logical to look about for other means whereby the end could be accomplished more subtly

For example, it would be reasonable that starting with benzaldehyde or p-hydroxybenzaldehyde, or phenylglyoxylic acid as proposed by Rosenmund and Dornsaft (20), and N methylglycine (sarcosine) the body could cause their condensation to form $\beta$ hydroxy N methylphenylalanine or its p-phenolic analogue This general reaction requires only a low order of activation *in vitro* and is in effect an aldol condensation, which has been invoked to account for a number of biochemical reactions

While this initial reaction and the subsequent ones (steps 1? and 2?) have no direct experimental substantiation, the resulting compound, N methyl phenylserine, should behave similarly to phenylalanine although possibly more

slowly with respect to enzymatic reactions in general, as outlined in the initial consideration of epinephrine synthesis If this be so, the decarboxylation of *l*-N-methyl-3,4-dihydroxyphenylserine by Holtz' *l*-dopa decarboxylase would give rise immediately to the naturally occurring form of epinephrine (step 3) Starting with glycine instead of sarcosine one could explain similarly the formation of arterenol, which has been claimed to be the substance responsible for the mediation of adrenergic nerve impulses (21) Even if the latter course of reactions should take place leading to the synthesis of epinephrine it would not preclude the occurrence in the body of those initially discussed up to and including the formation of β-(3,4-dihydroxyphenyl)ethylamine The fact that the subcutaneous injection of 3,4-dihydroxyphenylserine is reported not to cause an elevation of blood pressure in a dog (22) detracts no more from it as a precursor of epinephrine than the lack of pressor response when "dopa" is injected into a normal anesthetized animal (18a)

The recognition that the formation of various pressor agents having the basic β-phenylethylamine nucleus need not be direct steps in the formation of epinephrine, although such has been claimed to be the case (23), brings us to a consideration of these as causative agents in the pathogenesis of hypertension

HYPERTENSION That naturally occurring sympathomimetic amines may be etiologic agents in hypertension is more a revival of interest than a new concept In 1910 Ewins and Laidlaw commented in an introductory statement that the formation of tyramine from tyrosine in the intestine "has quite recently been regarded as playing a part in certain pathological states in which a high blood pressure is the most prominent symptom" (24) That same year Bain reported that tyramine was excreted in the urine of such patients but to a less extent than in patients having normal blood pressure (25), the implication being that it was retention of the amine that was responsible for the elevation of blood pressure The urohypertensine of Abelous and Bardier (26) was in part isoamylamine, and recently Lockett (27) has isolated pressor agents from normal urine Today the interest in the subject is the same, the emphasis on origin of these agents has changed

Holtz (18) and Blaschko (28) both observed that "dopa" was decarboxylated to form an amine which possessed both hyperglycemic and pressor properties (18c) Following their initial investigations but preceding Holtz' later work Bing pointed out, as based on his own *in vivo* experimentation, the hypothetical relation of "dopa" to essential hypertension (29)

He demonstrated that when "dopa" was injected into a kidney deprived of its blood supply or partially ischemic, but otherwise intact in a cat, and allowed to incubate for a suitable period of time, the re-establishment of circulation through that kidney resulted in an elevation of blood pressure This effect was interpreted as being due to the decarboxylation of "dopa" with the formation of the corresponding pressor amine in the ischemic kidney Moreover, the anoxic condition of that organ precluded the deamination of the pressor amine, as interpreted by Bing The explanation of these findings can be substantiated further by the citation of Holtz' report that he could demonstrate the formation

of β-(3,4-dihydroxyphenyl)ethylamine from "dopa" by the kidney or pancreas only when the organ was incubated with the amino acid in an atmosphere of nitrogen. This was necessary in order to block the oxidation of the resulting amine by amine oxidase (18a)

This acute experiment and its likely interpretation have led to the hypothesis that essential hypertension may be due to the decarboxylation of amino acid precursors of pressor amines in a damaged and ischemic kidney in which condition that organ is not capable of bringing about their deamination For such a circumstance to obtain it must be supposed that 1, the predominant site of inactivation of these amines is the kidney, 2, that a rather severe state of renal ischemia is an integral part of the hypertension, and 3, that the principal mode of inactivation of the phenolic pressor amines is by deamination Very likely none of these qualifications can be substantiated, except in an acute experiment where the amount of pressor amine liberated to the body on re-establishing circulation through the kidney is overwhelmingly greater than could be inactivated immediately by the body

The fact that the kidney is neither the only or even the major source of amine oxidase has been demonstrated by Bhagvat, Blaschko and Richter who found it to be widely distributed in the body (30) The fact that β phenyl n propylamine administered orally is inactivated by deamination and is not excreted as such unless a hepatotoxin, carbon tetrachloride, was previously administered, suggests the liver as a principal organ for deamination of that agent (31) It is difficult to believe that even under pathological conditions the rate of formation and liberation of such an agent by the kidney could exceed its rate of deamination by other organs Brown and Maegraith could demonstrate no reduction of amine oxidase in the livers of hypertensive rats (32) Actually, there appears to be no amine oxidase in the kidney of the rat (33) Certainly in the rat, an animal widely used for experimental hypertension, faulty deamination of pressor amines cannot be considered as contributing to the hypertension produced by damage to the kidney

It is doubtful whether a state of ischemia sufficient to abolish the functional capacity of an enzymatic oxidative system exists early in patients having essential hypertension, except in the rare instance of a lesion obstructing markedly the principal blood supply to the organ The decrease in renal blood flow and elevation of filtration fraction occurs earliest in the disease and appears to precede a decrease in the functional capacity of the renal tubules (34) This order of things would at least make less likely impaired oxidation of decarboxylated "dopa" the initiating factor for the increased tonus of the vascular system

The belief that phenolic pressor amines are inactivated in the body principally by deamination is not established as a certainty (35), although *in vitro* they can be shown to be deaminated rapidly by the enzyme The nature of this problem more properly relates to the inactivation of sympathomimetic amines, which will be discussed in another section of this review

*The alleviation of malignant hypertension* has been the purpose of a large amount

of research based on or interpreted in terms of the assumption that phenolic pressor amines are alterably related to the genesis of this disease Shroeder injected tyrosinase (phenol oxidase) into hypertensive rats and demonstrated a reduction in their blood pressure (36) Later he was able to extend this finding to some degree (37) His interpretation of these results was essentially that the damaged kidney was not capable of oxidizing the phenolic nucleus of the com pounds and that the added tyrosinase replaced the naturally occurrin g but now missing phenol oxidase This ignored the feeling of others that it is doubtful whether a true phenol oxidase capable of oxidizing tyramine or dihydroxyphenyl-ethylamine exists in mammalian tissues (38) Prinzmetal, Alles, et al came to the conclusion that the effect was nonspecific Their boiled preparation of tyrosinase was as effective in lowering the blood pressure of hypertensive patients as was their active material (39) Shroeder also reported that the administra-tion of crude amine oxidase preparations reduced the blood pressure of both hypertensive and normal rats (40), but these results are open to a similar criticism

Attempts have been made to associate the angiotonin or hypertensin theory of essential hypertension with the phenolic pressor amine hypothesis but these have not been successful While Croxatto and Croxatto did not make this distinction clear at first (41), they later showed that renal hypertensinase and amine oxidase were distinctly different enzymes as judged by enzyme characteristics (42) Bing, Zucker and Perkins also found angiotonin and amine oxidase to be different enzymes (43)

Still more recent is the interest in quinones as antipressor agents (44) There is no doubt that a large number of these agents, among them being 2-methyl-1,4-naphthoquinone which possesses vitamin K activity, reduce the mean blood pressure of acutely hypertensive rats without materially affecting the blood pressure of normal animals The rationale back of this effect is stated in part by most of these reports but is discussed in papers by Oster and his associates (45) It can be shown that if a solution of an amino acid or $\beta$-phenylisopropyl-amine is aerated with p-cresol and phenol oxidase or ascorbic acid, deamination of the compounds occurs (15, 46) Also, the first step in the oxidation of an ortho dihydric phenylethylamine by catechol oxidase is the corresponding ortho quinone (47) Such a quinone is very unstable, being further oxidized irrevers-ibly to melanin precursors or reduced to the corresponding catechol derivative Thus, if the ortho quinone and $\beta$-phenylethylamine were allowed to react, the quinone would be reduced and the $\beta$-phenylethylamine finally deaminated, giving rise to the corresponding aldehyde which could tie up another atom of $\beta$-phe-nylethylamine in a Shiff's base arrangement, as had already been suggested (48)

So far as we know, any substance which will tend to stabilize the catechol-quinone oxidation-reduction system, so as to prevent further oxidation, will inhibit the action of a phenol oxidase on the catechol derivative and so prevent the formation of the necessary quinone It has been reported that up to 1 gram of ascorbic acid per day for four months administered to hypertensive dogs did not reduce their blood pressure (49) This is interesting for it has been dem-

onstrated that not only does vitamin C deaminate β-phenylisopropylamine *in vitro* but the amount of the amine administered to dogs which was excreted in their urine was inversely related to the amount of ascorbic acid that was injected into the animals (46) Such experiments are not easily reconciled with the above theory

Unfortunately the quinones are ineffective insofar as they have been tried on human cases of hypertension (50) and on renal hypertensive dogs (51) In a personal communication from Doctors Goldring, Chasis and Smith they report that 2-methyl 1,4-naphthoquinone and 1,4-cyclohexandione in dosages up to 50 mgm /kgm were ineffective in the treatment of human essential hypertension (50) These compounds were reported by others as effective antipressor agents in rats Doctor Goldblatt has failed to observe a hypotensive effect in dogs with experimental renal hypertension which were treated with quinones, submitted to him for the test by some investigators who had observed a hypotensive effect in rats (51) Since the quinones are not nearly so effective anti pressor agents in those animals which are known to have amine oxidase normally present in their kidneys, it is difficult to relate their antipressor effect to the supplementation of restricted deamination in hypertensive rats having pernephric scarring but which never had the enzyme in their kidneys.

THE MODE OF ACTION OF SYMPATHOMIMETIC AMINES Much more is known about the manner in which the body affects these compounds than about the manner in which they affect the body There are probably as many reasons for this as there are approaches to the problem, and both have been and still are influenced by the state of knowledge of physiology or biochemistry of cellular metabolism To describe the action of epinephrine as being on or at the myoneural junction or on the neuro-effector mechanism carries a strong morphologic connotation. However, a recent publication by Marrazzi correlating the structure of sympathomimetic amines with their action on sympathetic ganglia as measured by alterations in postganglionic potentials is an attractive approach to this phase of the problem (52) Use of the terms neurotrophic and musculotrophic, or distinguishing between sympathomimetic and pseudosympathomimetic amines on the basis of the effect of these compounds as complicated by premedication of animals with agents (cocaine, ergotoxin, *etc* ) whose modes of action are no better understood, serves a very limited purpose

The efficacy with which epinephrine and its analogs acts is dependent on the functional integrity of the organs or systems directly and indirectly involved (53) The accelerating effect of epinephrine on glycogenolysis which is accompanied by an increase in cellular metabolism is a characteristic feature of its action This glycogenolytic effect is accompanied by hyperglycemia and an increase in blood lactate (54) but the amine does not seem to influence directly the utilization of an elevated blood sugar level (55)

This hyperglycemic characteristic of epinephrine transcends in sensitivity all generally recognized sympathomimetic effects of the agent (56), and is not approximated by its most closely related pressor amines The hyperglycemic effect of epinephrine is very definitely influenced by the adrenal cortical hormones

(53b, d) which synergize and by insulin which inhibits its effect (57) As the injection of sympathomimetic amines is increased from an infinitely small amount, the hyperglycemic response to epinephrine precedes its pressor effect (56) whereas the pressor action precedes whatever hyperglycemic effect the other sympathomimetic amines make manifest (58) These facts tempt one to wonder whether the latter agents produce an elevation in blood glucose except as they increase blood flow through the adrenal gland or otherwise indirectly influence the liberation of epinephrine

The theory of Gaddum and Kwiatkowski (59) for the mode of action of ephedrine introduced a unique concept which was extended by others (60) to cover the action of related compounds This hypothesis is based on the observation of Blaschko, Richter and Schlossman that ephedrine which is refractory to deamination by amine oxidase is able to inhibit the action of that enzyme on epinephrine by substrate competition (61) The formulation of the concept borrows precedent from the established competition between acetyl choline and eserine for choline esterase as the mode of action of eserine in the body (62) It was proposed, then, that ephedrine acted by inhibiting the normal inactivation by amine oxidase of epinephrine elaborated at nerve endings Thus the effect of epinephrine was increased and prolonged Since all sympathomimetic amines having a substituted isopropylamine side chain behave like ephedrine with respect to amine oxidase their pharmacological actions might be interpreted similarly The hypothesis serves to explain the potentiation of ephedrine to subsequent action of adrenergic nerves (59) and the increased pressor effect when epinephrine is injected following ordinary pressor doses of any of these isopropylamine derivatives (63) The diminishing pressor effect of successive ephedrine doses, and the ability of large doses of ephedrine and related compounds to diminish the response to epinephrine, were suggested as being due to blockade of the motor receptors by ephedrine (64) Thus large doses of ephedrine could inhibit the action of epinephrine while blocking its deamination by amine oxidase

The simplicity of this hypothesis at once makes it appealing However the theory is probably no more secure than the suggestion that amine oxidase is responsible for the inactivation of epinephrine in the body, which does not seem to be the case, and it does not account for the very great diversity of pharmacologic effects of these agents

The theory that the central stimulating action of amphetamine is due to the competitive inhibition of tyramine oxidation by amine oxidase thus diminishing the formation of aldehydes which depress cerebral oxidations (65) is inconclusive Many similar amines which do not possess this property of central stimulation likewise depress the *in vitro* oxygen uptake of brain tissue There appears to be no consistent difference between the depression of brain respiration *in vitro* by the optical forms of amphetamine used as a substrate nor is substrate competition materially influenced thereby, although there is a great difference in the central stimulating effect of the *d* and *l* forms of the compound (B)

Neither of these theories is concerned with the mode of action of epinephrine nor does either have as a major part of its thesis the possibility that the com-

pounds *per se* may be responsible predominantly for the effects they produce Certain recent developments permit the consideration of the "receptor mechanism" or "receptor substance" as an integral part of the mechanism for contraction, secretion, etc , or as a component of the metabolic cycle responsible for such action

For a number of reasons we have looked to an effect of the sympathomimetic amines on the carbohydrate and on the associated phosphorylation cycles as likely sites of their cellular action The physiologic response to epinephrine and the related compounds is a dynamic one affecting the principal function of a tissue, contraction in the case of muscle

During the past few years a great deal of research has related myosin to adenosinetriphosphatase (ATPase), and has associated the breakdown of adenosintriphosphate (ATP) with the alteration of bifringence of myosin and with muscular activity (66) Another function of the dephosphorylation of ATP is to furnish energy and phosphate for the phosphorylation of glucose (67), which process is necessary before the latter compound can undergo normal degredative procedures

Several points of attack suggest themselves for a study of the effect of sympathommetic amines on this trigger mechanism. Some of them have been subjected to investigation. 1 Preliminary experiments in our laboratory did not show the breakdown of ATP to ADP (adenosindiphosphate) in the presence of ATPase to be affected materially *in vitro* by these compounds This is not surprising, for under ordinary experimental conditions it would be difficult to note other than inhibitory effects 2 The rate or extent of phosphorylation of ADP might be effected Govier, Bergmann and Beyer found the phosphorylation of thiamin to be increased by certain sympathommetic amines (68) 3 The breakdown of the principal phosphate donors for the synthesis of ATP, i.e., 1,3 diphosphoglyceric acid and enol phosphopyruvic acid, might be influenced by these compounds 4 The oxidation of dicarboxylic metabolites which serve as a substantial source of energy for the phosphorylation of compounds (69) might be influenced by these amines Omitting for the moment the rôle of creatinine, a marked effect of the sympathommetic agents at any one of these points might be thought to have a similar end effect on the ATP ATPase mechanism Other points of attack have been omitted at present because of lack of evidence bearing on them

The phosphorylation of thiamin in the presence of the succinate system has served as a model for determining the effect of sympathomimetic amines on the utilization of energy from succinate oxidation for phosphorylation processes *It has* been demonstrated that epinephrine and certain other sympathommetic amines are able to accelerate the oxidation of succinate and the utilization of that energy for the phosphorylation of thiamin (68b) The effect on succinate oxidation could not be demonstrated when the substrate was being oxidized in a complete and uninhibited set up, but when α-tocopherol phosphate was added to retard succinate oxidation the acceleration effect of these amines was striking Very likely the tocopherol phosphate served to reduce the rate of succinate oxidation from its maximum to that more resembling the normal

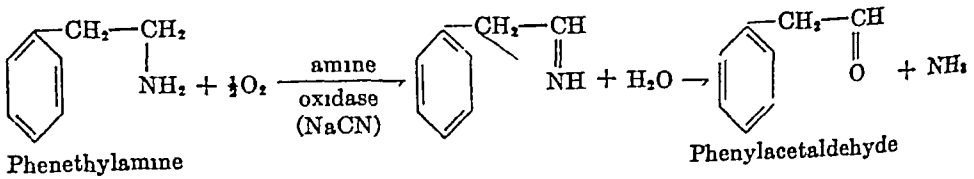
state *in vivo* Still more recently these investigators have found that certain of these amines accelerate the α-tocopherol phosphate-inhibited oxidation of lactate (70) Bearing on this point, Houchin and Mattill found that the addition of α-tocopherol phosphate made possible the reduction of the high succinoxidase activity of vitamin E deficient muscle to normal values (71) The prevalence of this state of partial inhibition is manifested in the tonus of muscle Much less energy would be required to alter in either direction this balanced system than need be expended otherwise

From the above lead it would be feasible to extend such research to cover the effect of the amines on the formation of ATP as influenced by the rate of succinate and other metabolite breakdown and the efficiency with which the energy is used This last point is a most important one, for Potter has recently stressed the fact that a high rate of oxygen uptake from succinate oxidation is no guarantee that phosphate-bond energy is thereby provided (72) Such evidence might assist materially to explain the mode of action of sympathomimetic amines Such a concept would allow much more flexibility than would be permissible if the principal actions of the amines were mediated through a common agent, epinephrine

THE INACTIVATION OF SYMPATHOMIMETIC AMINES The inactivation of sympathomimetic amines seems to be much better understood than is their mode of action Reviews bearing on this subject are those of De Meio (73), Beyer and Morrison (4e) and Hartung (74)

*Amine oxidase* is probably the enzyme most widely implicated in the inactivation of these compounds It was first described by Hare (75) and was partially purified by Kohn (76) The enzyme is widely distributed in mammalian tissue (30) from which it has not been isolated completely, is cyanide insensitive but can be inhibited by urea and phenylhydrazine *in vitro* (77) It is probably the same as tyramine oxidase and adrenalin oxidase (78) and may be the same as aliphatic amine oxidase (79) It has been proposed that the name monamine oxidase be used to distinguish it from diamine oxidase (80) Some of the discrepancies in the literature relating structure to rate of deamination of these compounds undoubtedly stem from the manner of preparing the enzyme for the *in vitro* experiments These differences are so great in many instances, notably in the case of β-phenylethylamine, as to warrant further investigation in an attempt to isolate and characterize the enzyme or enzymes

Amine oxidase brings about the deamination *in vitro* of a large number of derivatives of β-phenylethylamines, including many having a mono or dihydric nucleus (78, 4e, 81) In the course of deamination the compound is converted into the corresponding aldehyde which may be oxidized further to the analogous phenylacetic acid unless this later step is blocked, as by cyanide The course of this reaction is illustrated by the following equation




Phenethylamine Phenylacetaldehyde

Several alterations which may be made in the side chain render the molecule partially or totally refractory to deamination These include (1) Positioning the amino group on any other than the terminal carbon atom (78 81a) (2) The substitution of a single methyl group on the $\beta$-carbon atom in $\beta$ phenyl ethylamine does not abolish deamination, but increasing the length of that alkyl substitution or adding a second methyl group to make it a $\beta\beta$-dimethyl alteration does render the compound refractory to deamination (4e) (3) Likewise, the substitution of a single methyl group on the nitrogen atom is without qualitative effect whereas increasing the length of the substitution or the presence of two methyl groups on the nitrogen atom destroys or greatly retards the ability to be deaminated by amine oxidase (78, 81b, 4e) If the position of a single hydroxyl group on the nucleus of $\beta$ phenylethylamine be shifted from the para to the ortho position, the rate of deamination is markedly reduced (81b) The substitution of any of a number of polar groups other than hydroxyl or amino in the para position of the compound diminishes or completely inhibits deamination (B)

The presence of amine oxidase in the body, and particularly in the liver, determines whether pharmacologically active derivatives of $\beta$-phenylethylamine are effective and are excreted as such when administered by mouth Those derivatives wherein the amino group is on the terminal carbon atom of the side chain are deaminated in the body and are neither active nor excreted as such unless a hepatotoxic agent has been administered previously (31) Those compounds which have the amino group removed from the terminal position on the side chain are not deaminated to any great extent and are active when taken orally For the most part, they are excreted as such by man and the dog (7, 31) and to a lesser extent by rabbits (83) and rats (84)

Within rather narrow limits of structural variability there seems to be some relationship between pressor activity and rate of deamination, among those compounds which are deaminated, and also a relation between duration of action and whether or not the compounds are deaminated More probably these are two distinct circumstances which appear to parallel over a short range, than to be causally associated If one transgresses these very narrow limits such a relationship becomes difficult to predict or interpret Some compounds related to $\beta$-phenylethylamine are deaminated by the enzyme and also are active pressor agents, others are deaminated but exert no pressor properties (Marfanil, 85), some are not deaminated and have good pressor qualities of long duration, while others are neither deaminated nor active as pressor amines (B)

Except for a delineation of *in vitro* results reference to mono or dihydric derivatives of $\beta$ phenylethylamine has been omitted, for amine oxidase probably plays no great rôle in their inactivation in the body (76, 86)

*The ascorbic-dehydroascorbic acid system* for the deamination of sympathomimetic amines was discovered during a search for the mechanism responsible for the inactivation of that amount of amphetamine and related compounds which was not excreted as the amine The reaction for the deamination of amphetamine is illustrated in the following equation

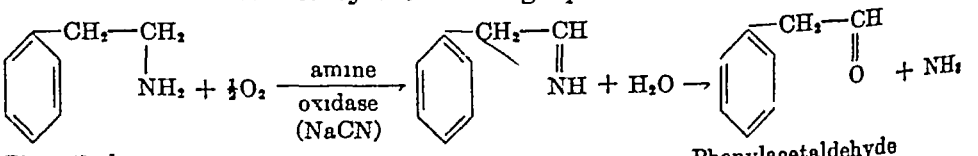
state *in vivo* Still more recently these investigators have found that certain of these amines accelerate the $\alpha$-tocopherol phosphate-inhibited oxidation of lactate (70) Bearing on this point, Houchin and Mattill found that the addition of $\alpha$-tocopherol phosphate made possible the reduction of the high succinoxidase activity of vitamin E deficient muscle to normal values (71) The prevalence of this state of partial inhibition is manifested in the tonus of muscle Much less energy would be required to alter in either direction this balanced system than need be expended otherwise

From the above lead it would be feasible to extend such research to cover the effect of the amines on the formation of ATP as influenced by the rate of succinate and other metabolite breakdown and the efficiency with which the energy is used This last point is a most important one, for Potter has recently stressed the fact that a high rate of oxygen uptake from succinate oxidation is no guarantee that phosphate-bond energy is thereby provided (72) Such evidence might assist materially to explain the mode of action of sympathomimetic amines Such a concept would allow much more flexibility than would be permissible if the principal actions of the amines were mediated through a common agent, epinephrine

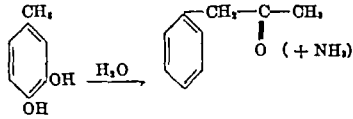
THE INACTIVATION OF SYMPATHOMIMETIC AMINES The inactivation of sympathomimetic amines seems to be much better understood than is their mode of action Reviews bearing on this subject are those of De Meio (73), Beyer and Morrison (4e) and Hartung (74)

*Amine oxidase* is probably the enzyme most widely implicated in the inactivation of these compounds It was first described by Hare (75) and was partially purified by Kohn (76) The enzyme is widely distributed in mammalian tissue (30) from which it has not been isolated completely, is cyanide insensitive but can be inhibited by urea and phenylhydrazine *in vitro* (77) It is probably the same as tyramine oxidase and adrenalin oxidase (78) and may be the same as aliphatic amine oxidase (79) It has been proposed that the name monamine oxidase be used to distinguish it from diamine oxidase (80) Some of the discrepancies in the literature relating structure to rate of deamination of these compounds undoubtedly stem from the manner of preparing the enzyme for the *in vitro* experiments These differences are so great in many instances, notably in the case of $\beta$-phenylethylamine, as to warrant further investigation in an attempt to isolate and characterize the enzyme or enzymes

Amine oxidase brings about the deamination *in vitro* of a large number of derivatives of $\beta$-phenylethylamines, including many having a mono or dihydric nucleus (78, 4e, 81) In the course of deamination the compound is converted into the corresponding aldehyde which may be oxidized further to the analogous phenylacetic acid unless this later step is blocked, as by cyanide The course of this reaction is illustrated by the following equation

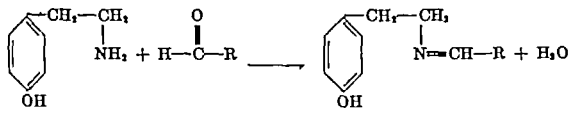



Phenethylamine Phenylacetaldehyde



The principle is similar to that reported by others In the presence of p-cresol, phenol, or hydroquinone, phenol oxidase is capable of oxidizing glycine, phenylalanine and leucine with the liberation of ammonia (87) There is no direct evidence attesting to the physiologic significance of this system *in vivo*

*Aldehydes, as agents for the deamination of sympathomimetic amines* have been proposed by Oster and his associates (45) The principle is that aldehydes formed in the body either by deamination or otherwise are capable of forming Shiff bases with the amines and so inactivating them Bernheim had previously suggested that such a reaction occurred in the course of deamination of amines by amine oxidase (48) The general course of the reaction is as follows



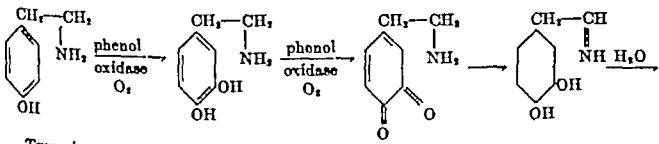
Tyramine Aldehyde Shiff base

This hypothesis lacks direct experimental support

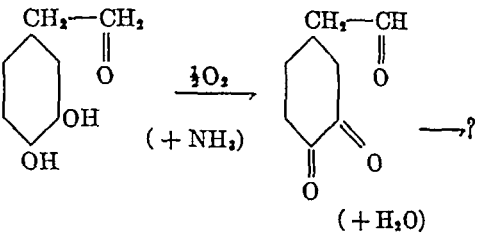
*The phenol oxidases* have received a tremendous amount of attention from a number of sources They have already been mentioned under a synonym, tyrosinase, in the sections on hypertension Most of the older ideas of epinephrine inactivation had to do with the oxidation of its catechol nucleus by such an enzyme.

There is no doubt that a number of such enzymes exist The names phenol oxidase or cresolase and polyphenol oxidase or catechol oxidase serve to distinguish between them on a basis of substrate affinities, although the two forms almost always occur together These enzymes can be obtained in a highly purified state Copper is the oxidation reduction component and the enzymes are therefore cyanide sensitive (88)

In the presence of phenol oxidase an atom of oxygen is introduced into such a molecule as tyramine adjacent to the hydroxyl group to form a catechol nucleus which in turn is oxidized to an ortho-quinone stage in the formation of melanin The course of the reaction is believed to be as follows



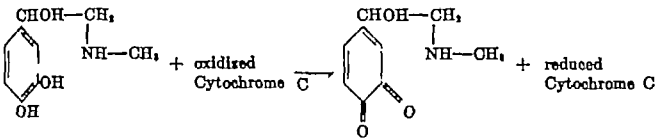
Tyramine ( + $H_2O_2$) ( + $H_2O_2$)




Reports dealing directly with the relation of structure to the oxidation of monohydric and dihydric phenylethylamines are those of Beyer (89), Alles (90), and Randall and Hitchings (91) The principal generalizations which can be derived are (1) Whereas p-hydroxyphenylethylamines may be rapidly oxidized, the corresponding meta and ortho hydric compounds are refractory to oxidation on the ring According to Randall and Hitchings (91) β-(m-hydroxyphenyl)-ethylamine is very slowly oxidized (2) If a hydroxl group is introduced on the same carbon atom of the side chain as that bearing the p-phenolic nucleus, the rate of oxidation of the nucleus is very greatly reduced Furthermore, if a keto group is substituted for the hydroxyl group on the β-carbon atom of the side chain, oxidation of the nucleus is abolished (3) While somewhat the same generalizations described in (2) obtain for the compounds having a catechol nucleus the differences in rate of oxidation are not nearly so distinct

The functional significance or even the presence of phenol oxidases in mammalian tissues is doubtful Bloch in 1917 (92), claimed to have demonstrated the "dopa" reaction in skin in experiments wherein "dopa" was incubated with the tissue and over a period of time melanin was formed Similarly, Pugh (93) demonstrated the "dopa" reaction in the skin of rabbits and the report was confirmed by Charles (94) Cadden and Dill have reported a polyphenolase extracted from kidney but it was more of the nature of a laccase and did not oxidize compounds of the nature with which we are concerned (95) Hageboom and Adams reported the presence of a phenolase in a mouse melanoma which oxidized tyramine (96) This is the closest to normal tissue in which such an enzyme has been shown to be present Bhagvat and Richter have surveyed tissues from a large number of animal species and concluded that a phenol oxidase could not be demonstrated in mammalian tissues by the usual methods (38) Aside from the doubtful presence and lack of functional significance of a phenol oxidase for the inactivation of sympathomimetic amines in the mammalian body there are a number of physiological observations which support this view These will be considered in the section pertaining to the conjugation of phenolic amines

*The cytochrome-cytochrome oxidase* system which is so universally present in tissues has been shown repeatedly to oxidize epinephrine and related compounds having an ortho dihydroxy phenolic nucleus, *in vitro* (97) The equation for the reactions may be written as follows




However, it is doubtful whether it functions in this manner in the body Hydroquinone, catechol, and homogentistic acid which are oxidized by cytochrome C *in vitro* are excreted as such or in a conjugated form

*Conjugation* appears to be the principal mode of inactivation of phenolic sympathomimetic amines The evidence for this is strong but lacks something of the finality of the correlation of deamination by amine oxidase with inactivation or excretion of $\beta$ phenylethylamines and $\beta$-phenylisopropylamines

The fate of monophenolic sympathomimetic amines has been particularly difficult to elucidate, owing to a lack of specific analytical methods The first noteworthy study of the excretion of tyramine, $\beta$-(p-hydroxyphenyl)ethylamine was that of Ewins and Laidlaw who administered 500 mgm. of the material to dogs and recovered up to 25 per cent of the ingested material as p hydroxyphenylacetic acid. Perfusion through liver and uterus oxidized tyramine to p-hydroxyphenylacetic acid, but perfusion through the heart completely destroyed the amine (98) These perfusion experiments anticipated the report of Bernheim and Bernheim that the abilities to deaminate, oxidize or conjugate tyramine, tyrosine and phenol *in vitro* were variously and independently present in different tissues Cardiac muscle was capable of opening up the ring in these molecules (99) The administration of such large quantities of the amine as was undertaken in the above experiments (98) permits a certain reservation as to the physiologic significance of the results

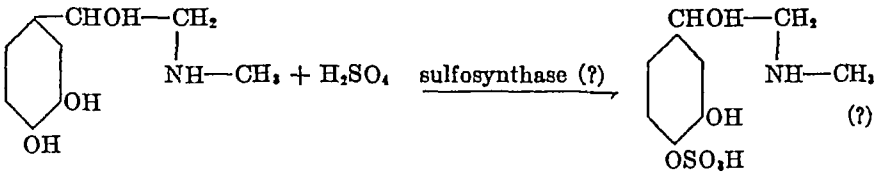
Beyer and Stutzman (100) administered 30 to 90 mgm doses of tyramine and $\beta$(p-hydroxyphenyl)isopropylamine to both man and dog While they did not notice any definite signs of oral activity they concluded that the inactivation of these compounds must be of such a nature as not to violate inalterably the integrity of the molecule, as would be the case for an oxidative process This was based on the fact that following oral administration there was excreted in the urine a compound which was identical with the one ingested, as judged by pressor activity and oxidation by phenol oxidase and amine oxidase These results might be quite reconcilable with those of Ewins and Laidlaw if measures for quantitation of results in the two instances were more satisfactory

Just what the nature of this conjugation is remains to be established Conjugation at either the amino or the phenolic group renders tyramine inactive as a pressor agent (B) Barger and Dale found acetoxyphenylethylamine inactive on intravenous injection (101) Loeper reported the synthesis of the sulfuric

acid ester of tyramine (102) and found it to possess no pressor activity in the cat unless first hydrolyzed to yield tyramine (103) To my knowledge their synthesis of the ester has not been repeated successfully

Arnolt and De Meio have reported that the conjugation of phenol in liver and intestinal slices is an enzymatic process They concluded that the process, which in itself does not involve the consumption of oxygen, requires for its activation the energy produced by reactions coupled with oxygen consumption (104) Williams has studied the relation of *o*, *m* and *p* substitutions to the conjugation of phenols (105)

Conjugation of sympathomimetic amines having a catechol nucleus is more clearly defined Richter (106) and Richter and McIntosh (107) reported that an enzyme, sulfosynthase, was responsible for the conjugation of sulfuric acid with the catechol nucleus Apparently the over-all reaction is as follows, although there is no evidence that conjugation necessarily is in the para position or that only one phenolic group is involved




It was clear that when epinephrine, β(3,4-dihydroxyphenyl)ethylamine and β(3,4-dihydroxyphenyl)-β-hydroxyisopropylamine were administered orally these investigators were able to demonstrate in urine only a conjugated form of the compound which after hydrolysis yielded the original agent as analyzed chemically or biologically Their work has been confirmed and extended recently by Beyer and Shapiro (108) Both groups of investigators are agreed that the majority of the drug administered thus is excreted in a conjugated form Qualitatively this is independent of the mode of administration The last is an important point and one which will need further study before this can be considered unequivocally the dominant mode of inactivation of these agents The reason for this is that the intestine and liver are the principal sites of phenol conjugation of epinephrine

Several investigators have studied recently the conjugation of phenols with sulfuric acid (109)

Before ceasing the discussion of conjugation as a mode of inactivation it might be well to point out that on the whole conjugation of the amino group has been neglected, possibly prematurely Snyder, Goetze and Oberst have just reported that the rat is capable of conjugating ephedrine and β-hydroxy-β-methyl-β-phenylethylamine The total recovery of ephedrine was increased by as much as 40 per cent and the latter compound by as much as 80 per cent when the urine of the animals was hydrolyzed (84) Although this may be a species characteristic, the observation is an interesting one

*Renal excretion of sympathomimetic amines* is dependent on their mode of

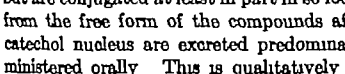
inactivation in the body Thus this section can be made to serve as a summary of the salient points in the preceding paragraphs

If the nucleus is unsubstituted by hydroxyl groups, derivatives of phenylethylamine which have the amino group on the terminal carbon atom are deaminated in the body and are not excreted as such. Where the amino group is not on the terminal carbon atom the compound is refractory to deamination, except by systems ordinarily of secondary importance, and are excreted for the most part as such Para hydroxy derivatives of $\beta$ phenylethylamine, regardless of the position of the amino group on the side chain, are inactivated in the body but are conjugated at least in part in so loose a manner as to be indistinguishable from the free form of the compounds after excretion. Compounds having a catechol nucleus are excreted predominantly in a conjugated form when administered orally This is qualitatively independent of the mode of administration. On hydrolysis of the urine the free form of the sympathomimetic amine is hydrolyzed from its conjugate

THE RELATION OF STRUCTURE TO PHARMACOLOGIC ACTION The purpose of this section is to indicate in general the quantitative and qualitative changes in pharmacologic properties that have been found to accompany basic and systematic alterations in the structure of sympathomimetic agents In some instances these compounds are so divergent in structure as to have in common only certain pharmacologic properties, in other instances the alterations in function induced by the compounds are so at variance that their chemical structures are the greatest point of similarity

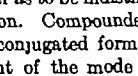
The following formulae illustrate several of the type structures and alterations which will be discussed in this section The list is not complete but may serve as a guide to those not familiar with the nomenclature of such compounds

*Aliphatic amines*

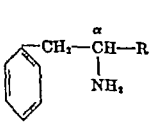


2-Aminoheptane "Tuamine"

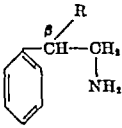
*Phenethylamine*



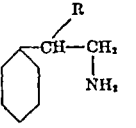
*Modifications of the phenethylamine side chain*



R = $C_nH_{2n+1}$ etc
$\alpha$ — substitution



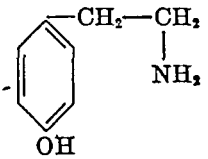
R = $C_nH_{2n+1}$ etc
$\beta$ — substitution



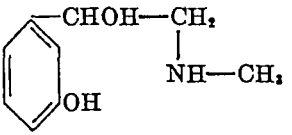
R = OH or O
aliphatic hydroxyl or keto substitution

*Substitutions into the phenyl nucleus*

a Monohydroxyphenylethylamines

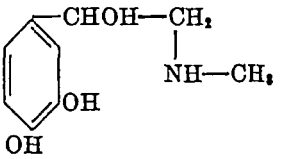


"Tyramine" para-hydroxyl radicle



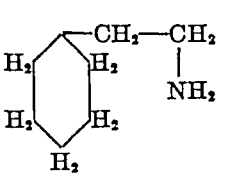
"Neosynephrin" meta-hydroxyl radicle

b Dihydroxyphenylethylamines

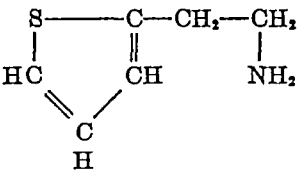


"Epinephrine" 3-4 dihydroxy substitution

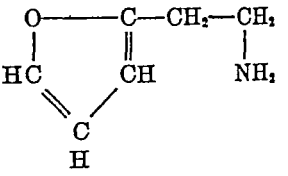
*Other types of aromatic nuclei*



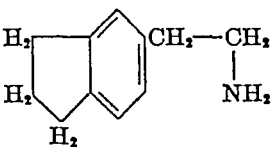
β-cyclohexylethylamine



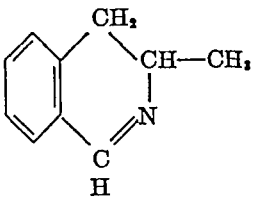
β-(2-thienyl)ethylamine



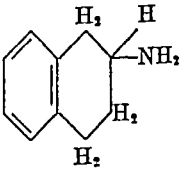
β-(2-furyl)ethylamine



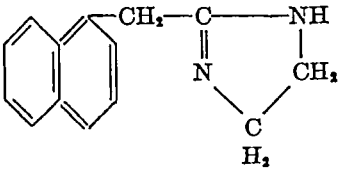
β-(5-hydrindenyl) ethylamine



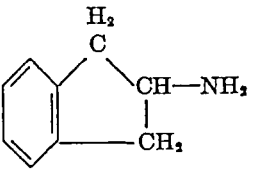
3-methyl-3,4 dihydroisoquinoline



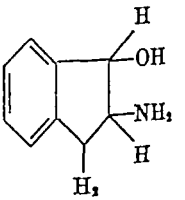
β-tetrahydronaphthylamine



2-naphthyl-(1')-methyl imidazoline "Privine"



2 aminoindane



1-hydroxy-2-aminohydrindene

*Aliphatic amines* The recent reports on aliphatic amines, as vasoconstrictor (110) and as antispasmodic agents (111), have served to renew interest in this group of compounds Actually the first critical investigation of a series of 21 alkylamines was that of Barger and Dale (101) Their series included the normal monoalkylamines, a number of iso-monoalkylamines from methylamine to undecylamine, and representative di, tri and quartenary ammonium compounds The lowest members of the group were found to be depressor agents or to have no effect on blood pressure (101, 112) Definite pressor activity was attributed to n-butylamine, maximal pressor activity of the compounds was resident in

n-hexylamine For compounds of greater chain length pressor activity decreased progressively although toxicity increased

Rohrmann and Shonle have synthesized and studied pharmacologically (113) the most systematic group of aliphatic pressor amines to date They confirmed the observation of Barger and Dale that, of the n l alkylamines, l aminohexane had the greatest pressor effect. Positioning of the amino group on the second carbon atom of heptane or octane gave better pressor results than substitution on any other carbon atom 2-Amino-4-methyl hexane, reportedly the most active compound of their series, and 2-amino-4-methyl heptane were more active than either of their 3-methyl or 5-methyl counterparts

As a pressor agent the best of the aliphatic amines is less active than ephedrine (113b) and they all demonstrate tachyphylaxis (101, 110c, 114)

*The phenylethylamine structure* Recalling that 1-aminohexane has a fair degree of pressor activity it is interesting that cyclohexylamine possesses only about $\frac{1}{3}$ its pressor potentialities Cyclohexylmethylamine is only slightly more active than cyclohexylamine, but when the amino group is further removed from the ring by one carbon atom the resulting $\beta$-cyclohexylethylamine has an activity sixfold greater than cyclohexylmethylamine and almost as great as for ephedrine $\beta$-cyclopentylethylamine has practically the same pressor activity as $\beta$-cyclohexylethylamine (113b) There is another interesting point in these data of Rohrmann and Shonle that bears reiteration at this time 2 Amino-4-methylhexane and 2-amino-4-methylheptane were about four times as active as 2-amino-3-methylhexane and 2 amino-3 methyl heptane, although all the compounds were less active than $\beta$-cyclopentylethylamine or $\beta$-cyclohexylethylamine. In both types of compound the amino group is one carbon atom removed from that bearing the ring, or from the actual branching of a long chain

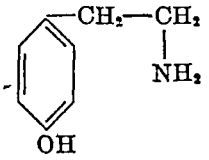
Barger and Dale (101) came to the conclusion that the aromatic nucleus should be one carbon atom removed from the amino group on the side chain Whereas aniline had no pressor effect, benzylamine was slightly active $\alpha$-Phenylethylamine was still more active while $\beta$-phenylethylamine had maximal activity and $\gamma$ phenylpropylamine was again less active Although there have been many substantiations of this fundamental point one of the best was that of Hartung and Munch (115) who studied the pressor effects of four isomeric phenylpropylamines Actually this beta relationship of the amino group to the aromatic nucleus transcends this group of compounds and appears in other series as well

*Modification of the aliphatic side chain* If one substitutes an alkyl chain, exceeding a methyl group, or an aryl group on either the alpha or beta carbon atom the sympathomimetic effect of the resulting agent is markedly reduced, abolished or reversed This has been found to be true for substitutions in the beta position with respect to the amine by Warren et al (116), and Beyer (B) using compounds of the phenylisopropyl series synthesized by Suter and Weston (117)

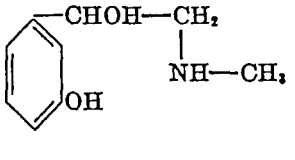
Schaumann found that if the side chain of epinephrine was increased to four carbon atoms, the compound was no longer pressor but was depressor in char

*Substitutions into the phenyl nucleus*

a Monohydroxyphenylethylamines

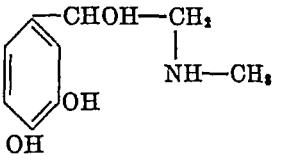


"Tyramine"
para-hydroxyl
radicle



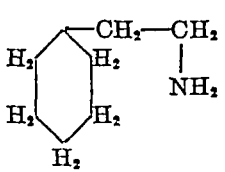
"Neosynephrin"
meta-hydroxyl
radicle

b Dihydroxyphenylethylamines

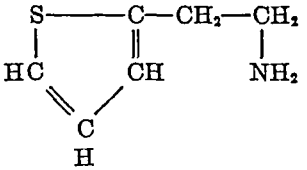


"Epinephrine"
3-4 dihydroxy
substitution

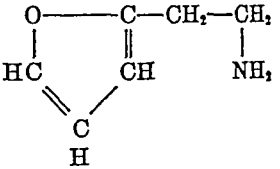
*Other types of aromatic nuclei*



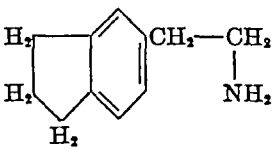
β-cyclohexylethylamine



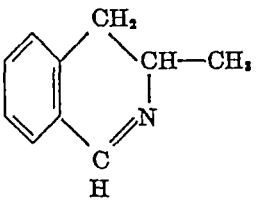
β-(2-thienyl)ethylamine



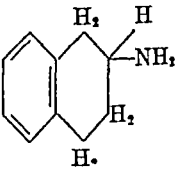
β-(2-furyl)ethylamine



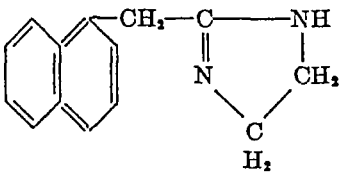
β-(5-hydrindenyl)
ethylamine



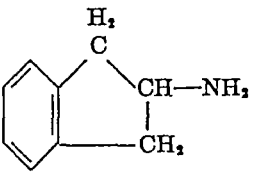
3-methyl-3,4 di-
hydroisoquinoline



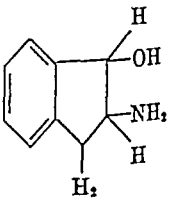
β-tetrahydro
naphthylamine



2-naphthyl-(1')-
methyl imidazoline
"Privine"



2 aminoindane



1-hydroxy-2-amino-
hydrindene

*Aliphatic amines* The recent reports on aliphatic amines, as vasoconstrictor (110) and as antispasmodic agents (111), have served to renew interest in this group of compounds Actually the first critical investigation of a series of 21 alkylamines was that of Barger and Dale (101) Their series included the normal monoalkylamines, a number of iso-monoalkylamines from methylamine to undecylamine, and representative di, tri and quartenary ammonium compounds The lowest members of the group were found to be depressor agents or to have no effect on blood pressure (101, 112) Definite pressor activity was attributed to n-butylamine, maximal pressor activity of the compounds was resident in

(desoxyephedrine, pervitin) which stimulates the central nervous system to about the same or a greater extent than does β-phenylisopropylamine, depending on the author (127c, 129), cinnamylephedrine is reported to have local anesthetic properties (130), and certain others are claimed to have some analgesic effect (131), the N-ethyl derivatives of ephedrine (127b) and epinephrine (132) are reported to have good bronchodilator effect although their pressor activities are considerably lower than those of the parent compounds

*Alteration of the aromatic nucleus* If a single hydroxyl group is introduced into the ring, the o-hydroxy compound is usually the least active, the m hydroxy the most active and the p-hydroxy derivative is intermediate but nearer the m-hydroxy in its potentialities The general order of pressor activity of these monohydric amines is on the whole much greater than for the nonphenolic compounds, ranging roughly from $\frac{1}{10}$ to $\frac{1}{100}$ as active as epinephrine for the phenolic and $\frac{1}{1000}$ to inactivity for the nonphenolic The observation of the relationship of position of the hydroxyl group to activity again goes back to the report by Barger and Dale (101) In the most recent report on the subject the relative inactivity of the ortho and the superior effectiveness of the meta hydroxy compounds are borne out (133) Although Barger and Dale did not appreciate the difference in activity of the meta and para compounds, a sufficiently large number of reports testify to the unequivocally greater activity of the meta hydroxy compounds (127d, 118, 134) In general the meta and para hydroxy compounds are more toxic than are their nonphenolic counterparts, they have a greater effect on intestinal activity, they are not as active by oral administration, they have a somewhat greater bronchodilator action, and they do not exhibit tachyphylaxis In all these respects the meta hydroxy compounds are superior Moreover the latter group more nearly simulates the actions of epinephrine and its related agents having a catechol nucleus Its pressor action is reversed by ergotamine and potentiated by cocaine (126) pancreatic secretion is stimulated (135), and, in the case of β(3 hydroxyphenyl) β-hydroxyethylmethylamine, it is the only type of highly active pressor agent which does not exaggerate the cardiac irregularities potentiated by cyclopropane anesthesia (136)

If two hydroxyl groups are introduced into the nucleus, the pressor activity of the compound depends on their relation to each other and to the side chain Dakin was impressed sufficiently by the appearance of the catechol nucleus in epinephrine to consider it to be the essential part of the epinephrine molecule (137) There is no doubt that for maximal effectiveness regardless of the nature of the side chain, the ortho dihydroxy substitution must be in the meta and para positions These are the so-called true sympathomimetic amines from a pharmacologic standpoint Their pressor ratios run roughly from equal, or better, to $\frac{1}{5}$ of that of epinephrine. None of them is active as a pressor or bronchodilator agent in moderate dosage except by parenteral administration, their duration of action is relatively brief, and they are quite unstable agents Some idea of the essentiality of the 3,4-dihydroxy nucleus may be gained by a comparison of epinephrine pressor ratios of a number of β(di hydroxyphenyl) propylamines re-

ported by Graham, Cartland and Woodruff (4f) 2,3 dihydroxy = 1/750, 2,4 dihydroxy 1/1100; 2,5 dihydroxy 1/4000, 2,6 dihydroxy = depressor, 3,4 dihydroxy = 1/40 and 3,5 dihydroxy = 1/120

Of all the sympathomimetic amines, arterenol, β(3,4-dihydroxyphenyl)-β hydroxyethylamine is undoubtedly more active than epinephrine It has been proposed to be the hypothetical "sympathin" (21)

The methoxy-phenylethylamines are among the most frequently studied of the compounds having substitutions in the aromatic nucleus The mono, di and tri methoxy and methylene-dioxy derivatives of the more common β-phenyl ethylamine and β-phenylisopropylamine structures have been made In general these compounds are more toxic and less active as pressor agents than are the corresponding phenolic or catecholic derivatives The derivatives of β-phenylisopropylamine and especially β-(3,4-methylenedioxyphenyl) isopropylamine initiate symptoms of central nervous system stimulation when introduced into rats, but none of these agents has gained acceptance The most comprehensive pharmacological studies of these compounds were undertaken by Gunn and his associates (138)

Two co-operative projects of considerable magnitude in this field are the pharmacologic actions described by Hjort (139) for 22 derivatives of N-methyl-β-phenylethylamine out of a group prepared by Buck (140), and the pharmacologic actions of the methoxy phenylpropylamine series described by Graham and Cartland (4f, 133), the compounds being synthesized by Woodruff and his associates (141) Graham and Cartland have reported that in some instances the methoxy compounds showed superior bronchodilator activity with less pressor effect than the corresponding hydroxy compounds Mescaline, β-(3,4,5-tri methoxyphenyl)-ethylamine, has most interesting central nervous system stimulating properties but no notable pressor action (142)

One other type of substitution into the nucleus deserves mentioning The β-aminophenylethylamines are an interesting group, although little is known of them They more nearly simulate the pressor activity and lack of toxicity of the phenolic type compounds than any other substitution into the nucleus Ephetonal, p-amino ephedrine, is less than half as toxic as ephedrine, and is a good pressor compound (132b, 143) Its effects on pancreatic secretion (135) and as a bronchodilator agent (132b) have been described A number of similar compounds have been reported in the literature (144) and on the whole they conform to the above generalization

Although a number of other groups have been introduced into the benzene ring, none of them yields compounds of any great interest These include the halogens (145), esters and alkyl groups (125, 146), nitro group (125), and a number of others Probably a fair index of the relative effect of a number of these substitutions into the nucleus on the pressor activity of a basic compound are the following epinephrine pressor ratios obtained under strictly comparable circumstances for substituted phenyl derivatives of β-phenyl-β-hydroxyisopropylamine (B) m-hydroxy 1 25, p-amino 1 45, o-fluoro 1 80, m-fluoro 1 102, p-hydroxy 1 103, m-chloro 1 133, the parent compound 1 185, p-fluoro 1 275, p-methyl 1 353, p-chloro 1 550, p-methoxy 1 800

The substitution of other aromatic nuclei for the benzene ring leads to compounds whose actions vary from pressor to no effect to strong depressor action A few examples will serve to illustrate this diversity The simplest alteration is from the benzene to the cyclohexyl ring Recent reports along this line are those of Rohrmann and Shonle (113b) and Lands, Lewes and Nash (147)

The cyclohexyl and cyclopentylethylamines and isopropylamines have slightly less pressor effect, are volatile, but do not have the pronounced central nervous system stimulating effect of their phenyl homologs The isosteres of the phenyl ethylamines are interesting compounds The closest are the $\beta$-thienylethylamines These have been studied by Tainter (140b), Alles and Feugen (148), Warren, Marsh, Thompson, Shelton and Becker (116), and Van Zoeren (149) Of all the heterocyclic derivatives of the ethylamines these more nearly resemble the $\beta$-phenylethylamines pharmacologically Replacement of the sulfur by oxygen in the heterocyclic ring yields the $\beta$-furylethylamines (148) which are less active than the $\beta$-thienylethylamines One other heterocyclic compound which has the same pressor ratio as $\beta$ phenylethylamine is $\beta$ (5-hydrindenyl) ethylamine (B)

Other types of nuclei which have not yielded compounds of noteworthy sympathomimetic effects are, $\alpha,\beta$-diphenylethylamines (150), isoquinolines (151), 1 hydroxy 2-aminohydrindene (152, 153), $\beta$ tetrahydronaphthalylamine (101), and indanamines (153) The most successful of all researches in this direction are those reported by Schols (4b) and recently by others (154) for certain imidazolines of which "Privine" is the best known example Histamine is an example of a heterocyclic substituted $\beta$-ethylamine which is an intensely depressor agent. On the other hand an analog of tryptamine the quarternary trimethyl indole-ethylamine iodide (155), like so many quarternary ammonium compounds (101, 156), is a pressor agent

Many more compounds more or less resembling the structure of sympathomimetic amines have been prepared and studied On the whole they have served no outstandingly useful purpose in this field and so will not be reviewed here Reference to them can be found in the chemical literature

## REFERENCES[1]

(1) Chen, K K. and C F Schmidt Ephedrine and related substances Williams & Wilkins Co , Baltimore Md , 1930
(2) Hartung, W H Ephedrine and related compounds Influence of structure on physiological activity Chem Rev 9 303, 1931
(3a) Cori C F and A D Welch The adrenal medulla J A M A 116 2590, 1941
(3b) Tainter M L The sympathomimetic amines as epinephrine substitutes J A M A 116 2769 1941
(3c) Dunker, M F W and W H Hartung Aliphatic amines—A review, Parts I and II J Am Pharm Assoc 30: 619 1941
(3d) Chen, K K Pharmacology Ann. Rev Physiol 7 677 1945
(4a) Gold H The clinical importance and uses of sympathomimetic amines Ind Eng Chem 37: 117, 1945

[1] In a number of instances the author has referred to work done in this laboratory which for lack of time has not yet been prepared for publication

(4b) Scholz, C R Imidazole derivatives with sympathomimetic activity Ind Eng Chem 37 120, 1945
(4c) Hartung, W H Beta-phenethylamine derivatives Ind Eng Chem 37 126, 1945
(4d) Tainter, M L Pharmacologic methods and objectives in research Ind Eng Chem 37 138, 1945
(4e) Beyer, K H and H S Morrison Relation of structure to deamination of sympathomimetic amines Ind Eng Chem 37 143, 1945
(4f) Graham, B E, G F Cartland and E H Woodruff Phenylpropyl and phenyl isopropyl amines Ind Eng Chem 37 149, 1945
(5) Rose, W C Physiol Rev 18 109, 1938
(6) Barger, G and G S Walpole J Physiol 389 343, 1909
(7) Richter, D Biochem J 32 1763, 1938
(8) Raper, H S Biochem J 26 2000, 1932
(9) Moss, A R and R Schoenheimer J Biol Chem 135 415, 1940
(10a) Euler, U S v and T Sjostrand Naturwissenschaften 31 145, 1943
(10b) Holtz, P Naturwissenschaften 25 457, 1937
(11a) Emerson, R L Beitr chem Physiol u Path 1 501, 1902
(11b) Heinsen, H A Hoppe-Seyler's Ztschr f physiol Chemie 245 1, 1936
(12a) Blaschko, H J Physiol 101 337, 1942
(12b) Drill, V A Proc Soc Exper Biol and Med 49 557, 1942
(13a) Hanke, M T and K K Koessler J Biol Chem 59 835, 1924
(13b) Epps, H M R Biochem J 36 242, 1944
(14a) Raper, H S Physiol Rev 8 245, 1928
(14b) Heard, R D H and H S Raper Biochem J 27 36, 1933
(14c) Arnow, L E J Biol Chem 120 151, 1937
(14d) Arnow, L E Science 87 308, 1938
(14e) Rothman, A Proc Soc Exper Biol and Med 44 485, 1940
(15) Beyer, K H J Pharmacol 76 149, 1942
(16) Heard, R D H and A D Welch Biochem J 29 998, 1935
(17) Medes, G Biochem J 26 917, 1932
(18a) Holtz, P and K Credner Arch exper Pathol u Pharmakol 199 145, 1942
(18b) Holtz, P and K Credner Arch exper Pathol u Pharmakol 200 356, 1942-43
(18c) Holtz, P, K Credner and C Strubing Hoppe-Seyler's Ztschr f physiol Chem 280 9, 1944
(19) Vinet, A Compt rend soc biol 210 552, 1910
(20) Rosenmund, K W and H Dornsaft Berichte 52 1734, 1919
(21) Greer, C M, J O Pinkston, J H Baxter and E S Brannon J Pharmacol 62 189, 1938
(22) Unpublished experiments at F Hoffman-La Roche and Co, Basel Quoted by M Guggenheim Die biogenen Amine, A Karger, Basel and New York, p 431, 1940
(23a) Schuler, W and A Wiedeman Hoppe-Seyler's Ztsch f physiol Chem 233 235, 1935
(23b) Devine, J Biochem J 34 21, 1940
(24) Ewins, A J and P P Laidlaw J Physiol 40 78, 1910
(25) Bain, W Lancet 178 1190, 1910
(26a) Abelous, J E and E Bardier J Physiol et Pathol generales 10 627, 1908
(26b) Abelous, J E and E Bardier J Physiol et Pathol generales 11 34, 1909
(27) Lockett, M F J Physiol 103 68, 1944
(28) Blaschko, H J Physiol 96 50P, 1939
(29a) Bing, R J Am J Physiol 132 497, 1941
(29b) Bing, R J and M B Zucker J Exper Med 74 235, 1941
(30) Bhagvat, K, H Blaschko and D Richter Biochem J 33 1338, 1939
(31) Beyer, K H and W V Lee J Pharmacol 74 155, 1942

(32) Brown G M and B G Maegraith Brit J Exper Path 22 108, 1941
(33) Oster, h. A and N C Schlossman J Cell and Comp Physiol 20 373 1942
(34a) Bradley, S E New England J Med 231 421 1944
(34b) Goldring W and H Chasis Hypertension and hypertensive disease Chapter IV The Commonwealth Fund New York 1944
(35a) Kohn, H O J Biochem 31 1693 1937
(35b) Richter D and A H Tinget J Physiol 97 265 1939
(36) Schroeder, H A Proc Soc Exper Biol and Med 44 172 1940
(37) Schroeder H A and M H Adams J Exper Med 73: 531, 1940
(38) Bhagvat K and D Richter Biochem J 32: 1397 1938
(39) Prinzmetal, M G A. Alles C Margoles, A Kayland and D S Davis Proc Soc Exper Biol and Med 50 288 1942
(40) *Schroeder H A Science 95 306 1942*
(41) Croxatto H and R Croxatto Proc Soc Exper Biol and Med 48 392 1941
(42) Croxatto, R H Croxatto and L Marty Proc Soc Exper Biol and Med 52: 64 1943
(43) Bing R J M B Zucker and W Perkins Proc Soc Exper Biol and Med 48: 372 1941
(44a) Friedman B S Soloway J Marrus and B S Oppenheimer Proc Soc Exper Biol and Med 51 195 1942
(44b) Schafer P W Proc Soc Exper Biol and Med 55 274 1944
(44c) Schwarz, H and W M Ziegler Proc Soc Exper Biol and Med 55 160 1944
(44d) Schwarz H and W M Zifgler Am J Physiol 143 177 1945
(45a) Oster K A Naturo 150 289 1942
(45b) Soloway S and K A Oster Proc Soc Exper Biol and Med 50 108 1942
(45c) Oster, K A and H Sobotka J Pharmacol 78 100, 1943
(45d) Oster, k. A and M G Mulinos J Pharmacol 80 132 1944
(46) Beyer k. H. J Pharmacol 71 394 1941
(47a) Happold, F C and H S Raper Biochem J 19 92 1925
(47b) Ball E G and T Chen J Biol Chem 102: 691 1933
(47c) Wagreich H and J M Nelson J Biol Chem 115: 459, 1936
(48) Bernheim M L C J Biol Chem 93: 299 1931
(49) Moss W G and G E Wakerlin Fed Proc 3: 34 1944
(50) Goldring W Personal communication
(51) Goldblatt H Personal communication
(52) Marrazzi, A S Submitted for publication
(53a) Bosquet F Compt rend soc biol 111 665 1932
(53b) Chobrmis k. E Papachriston and J Korkas Ann Paediat 154 22, 1939
(53c) Friedenwald J S and W Buschke Am J Physiol 143: 387, 1943
(53d) Ende, S Ztschr klin Med 136: 108 1939
(54a) Cori C F and G T Cori J Biol Chem 84: 683 1929
(54b) Cori C F and K W Buchwald Am J Physiol 95: 71 1930
(54c) Heinauer, A H and G T Cori J Biol Chem 105 691 1934
(54d) Cori C F Endocrinology 26 285 1940
(55a) Dill, D B R E Johnson and C Daly Am J Med Sci 198: 702, 1939
(55b) Courtice F C C G Douglas and J G Priestley Proc Roy Soc London B 127 41 1939
(55c) Conn, J W E S Conn and M W Johnston J Nutrition 19 (supp ) 16, 1940
(55d) Soskin S H E Essex J F Herrick and F C Mann Am J Physiol 118 328, 1937
(56a) Cori C F and G T Cori Proc Soc Exper Biol and Med 27 560 1930
(56b) Baudouin A H Benard, J Lewin and J Sallet Compt. rend soc biol 118 529 1935
(57) Cori G T C F Cori and k. W Buchward J Biol Chem 86 375 1930

(58) Anderson, R C and K K Chen J Am Pharm Assoc 23 290, 1934
(59) Gaddum, J H and H Kwiatkowski J Physiol 94 87, 1938
(60a) Kwiatkowski, H J Pharmacol 69 198, 1940
(60b) MacGregor, D F J Pharmacol 66 393, 1939
(60c) Lawrence, W C, M C Morton and M L Tainter J Pharmacol 75 219, 1942
(61) Blaschko, H, D Richter and H Schlossman Biochem J 31 2187, 1937
(62) Easson, L H and E Stedman Proc Roy Soc B 121 142, 1936
(63a) Munch, J C and W H Hartung J Am Pharm Assoc 19 356, 1929
(63b) Tainter, M L Arch Int Pharmacodyn et Therap 41 365, 1931
(63c) Morton, M C and M L Tainter J Physiol 98 263, 1940
(64a) Loewe, S Ztschr exper Med 56 271, 1927
(64b) Curtis, F R J Pharmacol 35 333, 1929
(64c) Orzechowski, G, W Gronemeyer and G Malorny Arch exper Path u Pharmakol 196 237, 1940
(65a) Mann, P J G and J H Quastel Nature 144 943, 1939
(65b) Mann, P J G and J H Quastel Biochem J 34 414, 1940
(66a) Engelhardt, V A and M N Linbimova Nature, London 144 668, 1939
(66b) Needham, D M Biochem J 36 113, 1942
(66c) Bailey, K Biochem J 36 121, 1942
(66d) Dainty, M, A Kleinzeller, A S C Lawrence, M Miall, J Needham, D M Needham and Shih-Chang Shen J Gen Physiol 27 355, 1944
(67a) Euler, H v and E Adler Ztschr physiol Chem 235 122, 1935
(67b) Meyerhof, O Naturwissenschaften 23 850, 1935
(67c) Colowick, S P and H M Kalckar J Biol Chem 148 117, 1943
(68a) Govier, W M, V Bergmann and K H Beyer J Pharmacol, 85 140, 1945
(68b) Govier, W M, V Bergmann and K H Beyer J Pharmacol, 85 143, 1945
(69a) Kalckar, H Biochem J 33 631, 1939
(69b) Colowick, S P, M S Welch and C F Cori J Biol Chem 133 359, 1940
(69c) Colowick, S P, M S Welch and C F Cori J Biol Chem 133 641, 1940
(69d) Ochoa, S J Biol Chem 138 751, 1941
(70) Govier, W M, V Bergmann and K H Beyer J Pharmacol, in press
(71) Houchin, O B and H A Mattell J Biol Chem 146 306, 1942
(72) Potter, V R Arch Biochem 6 439, 1945
(73) De Meio, R H Chem Prod 6 37, 1943
(74) Hartung, W H Ann Rev Biochem, 1946, in press
(75) Hare, M L C Biochem J 22 968, 1928
(76) Kohn, H J J Biochem 31 1693, 1937
(77) Bernheim, F J Biol Chem 133 485, 1940
(78) Blaschko, H, D Richter and H Schlossman Biochem J 31 2187, 1937
(79) Pugh, C E M and J H Quastel Biochem J 31 286, 1937
(80) Sumner, J B and G F Somers Chemistry and methods of enzymes Academic Press, Inc, New York, p 269, 1943
(81a) Beyer, K H J Pharmacol 71 151, 1941
(81b) Beyer, K H J Pharmacol 79 85, 1943
(81c) Alles, G A and E V Heegaard J Biol Chem 147 487, 1943
(82) Beyer, K H and J T Skinner J Pharmacol 68 419, 1940
(83) Jacobsen, E and J Gad Arch exper Path u Pharmakol 196 34, 1940
(84) Snyder, F H, H Goetze and F W Oberst J Pharmacol, in press
(85) Beyer, K H and W M Govier Science 101 150, 1945
(86) Richter, D and A H Tingey J Physiol 97 265, 1939
(87a) Pugh, C E M and H S Raper Biochem J 21 1370, 1927
(87b) Robinson, M E and R A McCance Biochem J 19 251, 1925
(87c) Hubard, S S J Biol Chem 126 489, 1938
(88a) Keilin, D and T Mann Proc Roy Soc B 125 187, 1938

(88b) Kubowitz F Biochem Ztschr 299 82 1938
(88c) Dalton, H R and J M Nelson J Am. Chem Soc 60 3085, 1938
(88d) Tenenbaum L E and H Jensen J Biol Chem 145 293 1942
(89) Beyer, K. H J Pharmacol 77 247 1943
(90) Alles G A, C L Bloim and P R Saunders J Biol Chem 144 757, 1942
(91) Randall, L O and G H Hitchings J Pharmacol 80 77 1944
(92) Bloch B Ztschr physiol Chem 100 226 1017
(93) Pugh C E. M Biochem J 27: 476 1933
(94) Charles, D R. Genetics 23 523 1938
(95) Cadden J F and L V Dill J Biol Chem 143 105 1042
(96) Hageboom G H and M H Adams J Biol Chem 145: 273 1942
(97a) Keilin, D and E F Hartree Proc Roy Soc B 125 171 1938
(97b) Blaschko, H and H Schlossman J Physiol 98: 130 1940
(98) Ewins A J and P P Laidlaw J Physiol 41 78 1910
(99) Bernheim F and M L C Bernheim J Biol Chem 153 369 1944
(100) Beyer, K. H. and J W Stutzman To be published
(101) Barger, G and H H Dale J Physiol 41 19 1910
(102) Loeper, M and J Parrod Bull soc chim biol 20 1117 1938
(103) Loeper M, J Loeper, A Lemaire J Cottet and J Parrod Compt rend soc biol 128: 1050 1938
(104a) Arnolt R J and R. H de Meio Rev soc argentina biol 17: 570 1941
(104b) Arnolt R. J and R H de Meio Rev soc argentina biol 30: 40 1942
(105) Williams R T Biochem J 32 878 1938
(106a) Richter, D J Physiol 98 25P, 1940
(106b) Richter, D J Physiol 98 361 1940
(107) Richter, D and F C MacIntosh Am J Physiol 135: 1 1941
(108) Beyer K H and S H Shapiro Am J Physiol 144: 321, 1945
(109a) De Meio, R. H Arch. Biochem 7: 323 1945
(109b) Bernheim F and M L C Bernheim J Pharmacol 78 394, 1943
(109c) Torda, C J Pharmacol 78 331 1943
(109d) Deichmann, W B Arch Biochem 3 345 1944
(110a) Proetz A. W Ann Otol, Rhinol and Laryngol 51 112 1942
(110b) Roman Vega D A. and J Adriani Anesth and Analges. 23: 248 1944
(110c) Jackson D E J Lab Clin Med 29 150 1944
(111a) Jackson D E J A. M A 106: 357, 1936
(111b) Gruber, C M J Pharmacol 56 284 1936
(112) Hauschild, F Arch Exper Path. u Pharmakol 201: 569 1943
(113a) Rohrman, E and H A Shonle J Am Chem. Soc 66: 1516, 1044
(113b) Rohrmann E and H A Shonle. 2-Aminoalkanes as pressor substances Presented before the Div of Med Chem, Am Chem Soc April 1944
(114) Ahlquist R P J Am Pharm Assoc 32: 151, 1943
(115) Hartung W H and J C Munch J Am Chem Soc 53 1875 1931
(116) Warren, M R D G Marsh, C R. Thompson R. S Shelton and T J Becker. J Pharmacol 79: 187, 1943
(117) Sutter C M and A W Weston J Am Chem Soc 64 583 1942
(118) Schaumann D Arch exper Path u Pharmakol 160 127 1931
(119) Hartung W H, J C Munch, W A. Deckert and F Crossley J Am Chem. Soc 52 3317, 1930
(120) Chen K. K C K Wu and E Hendriksen J Pharmacol 38 303, 1929
(121) Piness G., H Miller and G A. Alles J A M A 94 790 1930
(122) Gunn J A and M R Gurd J Physiol 98: 424, 1940
(123) Schultz J W E C Reif, J A Bacher, W S Lawrence and M. L. Tainter J Pharmacol 71: 62 1941
(124a) Alles G A and P K Knoefel. U Cal Pub in Pharmacol 1: 101, 1938

(124b) CRIMSON, J M AND M L TAINTER J Pharmacol 66 146, 1939
(125) BARGER, G Organic chemistry in biology and medicine McGraw-Hill Book Co, New York, 1930
(126) TAINTER, M L AND M A SEIDENFELD J Pharmacol 40 23, 1930
(127a) JACOBSEN, E, A WOLLENSTEIN AND J T CHRISTENSEN Klin Wchenschr 17 1580, 1938
(127b) BECKER, T J, M R WARREN, D G MARSH, C R THOMPSON AND R S SHELTON J Pharmacol 75 289, 1942
(127c) NOVELLI, A N AND M L TAINTER J Pharmacol 77 324, 1943
(127d) EHRHART, G Metallborse 20 1800, 1930
(128a) KONZETT, H Arch exper Path u Pharmakol 197 41, 1940
(128b) GEIGER, E Arch Internat Pharmacodyn et Therap, 6 64, 1939
(128c) STUTZMAN, J W AND O S ORTH J Pharmacol 69 1, 1940
(129a) GRAF, O Arbeitsphysiol 10 692, 1939
(129b) HAUSCHILD, F Arch exper Path u Pharmakol 191 465, 1939
(129c) SCHULZ, F AND S DECKNER Klin Wchnschr 21 674, 1942
(129d) IVY, A C AND F R GOETZL War Med 3 60, 1943
(130) SCHUITZ, F H AND P H BARBOUR J Pharmacol 76 295, 1942
(131) KULZ U S Patent 2,276,618 (Mar 17, 1942)
(132a) KONZETT, H Arch exper Path u Pharmakol 197 27, 1940
(132b) CAMERON, W M AND M L TAINTER J Pharmacol 57 152, 1936
(133) GRAHAM, B E AND G F CARTLAND J Pharmacol 81 360, 1944
(134a) HARTUNG, W H, J C MUNCH, E MILLER AND F CROSSLEY J Am Chem Soc 53 4149, 1931
(134b) TAINTER, M L Arch internat Pharmacodyn et Therap 42 128, 1932
(134c) SCHAUMANN, O Arch exper Path u Pharmacol 157 114, 1930
(134d) YOUMANS, W B, H F HANEY AND K W AUMANN Am J Physiol 130 190, 1940
(135) GREENGARD, H, R A ROBACK AND A C IVY J Pharmacol 74 309, 1942
(136) ORTH, O S, M D LEE, C H MELLISH AND J W STUTZMAN J Pharmacol 67 1, 1939
(137) DAKIN, H D Proc Roy Soc B 76 498, 1905
(138a) EPSTEIN, D, J A GUNN AND C J VIRDEN J Physiol 74 224, 1932
(138b) ELPHICK, G K AND J A GUNN J Physiol 81 422, 1934
(138c) GUNN, J A, M R GURD AND I SACHS J Physiol 95 485, 1939
(139) HJORT, A M J Pharmacol 52 101, 1934
(140) BUCK, J S J Am Chem Soc 54 3661, 1932
(141a) WOODRUFF, E H AND T W CONGER J Am Chem Soc 60 465, 1938
(141b) WOODRUFF, E H AND E PIERSON Ibid 60 1075, 1938
(141c) WOODRUFF, E H, J P LAMBOVY AND W E BURT Ibid 62 922, 1940
(141d) WOODRUFF, E H Ibid 64 2859, 1942
(142) RAYMOND-HAMET Arch exper Path u Pharmakol 169 97, 1932
(143a) DALMEN, O AND M OBERLIN German pat 521,729, Chem Abstr 25 3363, 1931
(143b) MAYER Drug Market 39 51, 1931
(144a) BOVET, D AND G BENOIT Compt rend soc biol 136 328, 1942
(144b) OBERLIN, M U S Pat 1,898,258, Feb 21, 1933
(144c) HAUSCHILD, F Klin Wchnschr 20 363, 1941
(144d) HARTUNG, W H AND C P FOSTER J Am Chem Soc, in press
(145a) EDKINS, R P AND W H LINNELL Quart J Pharm and Pharmacol 9 75, 1936
(145b) EDKINS, R P AND W H LINNELL Quart J Pharm and Pharmacol 9 203, 1936
(145c) HARTUNG, W H, J C MUNCH AND F S CROSSLEY J Am Chem Soc 57 1091, 1935
(145d) ZENITZ, B L AND W H HARTUNG Abstr Am Chem Soc p K-11 Apr 1939
(145e) SUTER, C M AND A W WESTON J Am Chem Soc 63 602, 1941
(146a) TAINTER, M L Quart J Pharm and Pharmacol 3 584, 1930

(146b) Ehrhart G Metalborse 20 1800, 1930
(147a) Gunn J A. and M. R. Gurd J Physiol 97:453 1940
(147b) Lands A M J R. Lewis and V L Nash. J Pharmacol 83 253, 1945
(148) Alles G A and G A Feigen J Pharmacol 72:265 1941
(149) Van Zoeren, G J U S Patent 2,367 703 (Jan 23) 1945
(150a) Tainter, M L, F P Luduena R W Lackey and E N Neubu J Pharmacol 77 317, 1943
(150b) Hjort, A. M J Pharmacol 50 131 1934
(157a) Hjort A M E J de Beer, J S Buck and L. O Randall J Pharmacol 76: 64, 1942
(151b) Ibid 76 71 1942
(151c) Ibid 76 252 1942
(151d) Ibid 76:258 1942
(151e) Ibid 76:263 1942
(152) Austein, W Arch exper Path u Pharmakol 169 114 1933
(153a) Levin N, B E Graham and H G Kolloff J Org Chem 9 380, 1944
(153b) Levin, N B E Graham and H G Kolloff Presented before the Med Chem. Div Am Chem Soc April 1944
(154a) Craver, B N, H F Chase and F F Yonkman J Pharmacol 82:275, 1945
(154b) Ogden, E and G A Emerson Fed Proc 4 132 1945
(154c) Warren, M R and H W Werner J Pharmacol 85 119, 1945
(154d) Emerson G A. J Pharmacol 82 42 1944
(154e) Yonkman, F F, B Rennick and H L Schwerma J Pharmacol 84:197, 1945
(155) Chen K K. and A L Chen J Am Pharm Assoc 22 813 1933
(156) Alles G A U Cal Pub in Pharmacol 2 161 1944

# THE FIXATION OF CARBON DIOXIDE AND THE INTERRELATIONSHIPS OF THE TRICARBOXYLIC ACID CYCLE

HARLAND G WOOD

*Division of Physiological Chemistry, Department of Physiology, University of Minnesota, Minneapolis*

CONTENTS

SECTION I INITIAL REACTIONS IN THE FIXATION OF CARBON DIOXIDE 199
1 $C_3$ and $C_1$ addition by oxalacetate $\beta$-carboxylase 200
2 $C_2$ and $C_1$ addition by the phosphoroclastic reaction 202
3 $C_5$ and $C_1$ addition by oxalosuccinate carboxylase 206
4 Summary 208
SECTION II MECHANISM OF FIXATION OF CARBON DIOXIDE IN DIFFERENT COMPOUNDS 208
1 $C_1$ compounds 208
2 $C_2$ compounds 209
3 $C_3$ compounds 213
4 $C_4$ compounds 214
5 $C_5$ compounds 215
6 $C_6$ compounds 216
7 Glycogen 216
8 Unidentified compounds 220
9 Summary 220
SECTION III THE TRICARBOXYLIC ACID OR KREBS' CYCLE 221
1 Evaluation of the experimental basis of the scheme 221
2 The tricarboxylic acid cycle and oxidation of carbohydrate 225
3 The tricarboxylic acid cycle and glycogen synthesis 228
4 The tricarboxylic acid cycle and oxidation of fatty acids 232
a The initial condensation reaction 232
b Evidence of the formation of $C_2$ compounds in the oxidation of fatty acids 236
c Formation of intermediary compounds of the cycle from labeled acetate and acetoacetate 240
5 Summary 242

It is proposed in this communication to consider the mechanism of carbon dioxide utilization by animal tissue together with those problems which are closely related to this subject The fixation of carbon dioxide occurs in the synthesis of glycogen and is intimately involved in biological oxidations through the tricarboxylic acid or Krebs cycle This cycle constitutes a mechanism by which carbohydrate and fat may be oxidized These closely interwoven problems make up the main task of this review

The early developments in this field do not need to be considered extensively since the material has been adequately reviewed by others (1), (2), (3) In the ten years which have elapsed since Wood and Werkman (4) first demonstrated carbon dioxide fixation by heterotrophic bacteria, there has been rather rapid advance This progress has been especially rapid since 1940, when general

agreement was reached that carbon dioxide can be used by most heterotrophic forms of life The acceptance of this phenomenon stemmed mainly from the fact that isotopes of carbon became available, which made possible the active study of utilization of carbon dioxide by organisms other than the special bacteria with which the first observations were made With isotopes it proved easy to demonstrate that many organisms can assimilate carbon dioxide, bacteria (5), (6), molds (7), protozoa (9), and animals (10), (11), (12), (13) After completion of this step, investigators were confronted with the more difficult task, that of solving the chemistry underlying the reactions by which the basic conversion of carbon dioxide to an organic linkage is brought about A good start has already been made in this direction but much still remains obscure Beyond the direct object of obtaining information concerning the chemical reaction lies the goal of interpreting the function of carbon dioxide fixation in biochemistry At present such considerations are largely speculation

The studies of carbon dioxide fixation have led, and will lead, not only to a better understanding of this process, but also, in an indirect way, to the solution of other problems. Already these investigations have contributed to the understanding of the mechanisms of the tricarboxylic acid cycle and of glycogen synthesis. Ultimately it is to be expected that the information will constitute a real contribution toward clarification of the chemical reactions of photosynthesis It is quite probable that the underlying reactions may be similar in the two processes, the essential difference being that the required energy is radiant in photosynthesis and chemical in the case of heterotrophic metabolism (14), (15), (16)

SECTION I INITIAL REACTIONS IN THE FIXATION OF CARBON DIOXIDE Until recently there was only one example of a reaction by which carbon dioxide was believed to be fixed in an organic linkage by heterotrophic forms of life. This reaction consisted of the combination of pyruvic acid and carbon dioxide, to form oxalacetate (17) The kinetics and intermediate steps of the reaction are still obscure Recently, in addition to this example of fixation by $C_3$[1] and $C_1$ combination, evidence has been presented of $C_2$ and $C_1$ addition (18), (16) and very recently of $C_4$ and $C_1$ combination (19) As yet $C_3$ and $C_1$ addition has not been demonstrated with animal tissues The biological significance of the last two reactions has received practically no consideration.

There is at present no reason to believe that other reactions of carbon dioxide fixation will not be observed, there are many decarboxylations which have not yet been tested for reversibility Certainly there is no longer any basis for assuming that $C_3$ and $C_1$ addition is the key reaction for all carbon dioxide fixation. The relative importance of the fixation reactions in biochemistry cannot be judged at present It is conceivable that some of the fixations are not essential for formation of necessary compounds, but that they are simply the result of the reversibility of breakdown mechanisms

There are three known examples of primary fixation reactions By primary fixation is meant the reaction in which the carbon to carbon linkage is first

[1] Compounds containing n number of carbons will be referred to as $C_n$

formed This is in distinction to the overall changes following this initial fixation of carbon dioxide Presumably the primary fixation reaction initiates fixation which ultimately leads to the inclusion of carbon from carbon dioxide in a large number of derived compounds

The three primary fixation reactions will be considered in order of their discovery

*$C_3$ and $C_1$ addition by oxalacetate β-carboxylase*

(1) $$C^{13}O_2 + CH_3\ CO\ COOH \rightleftharpoons C^{13}OOH\ CH_2\ CO\ COOH$$

Reaction 1 was first proposed as a working hypothesis for the fixation of carbon dioxide in the propionic acid fermentation (20) This proposal was based on the observation that in the fermentation of glycerol by the propionic acid bacteria the succinate formed and carbon dioxide utilized were approximately equimolar This fact pointed to a union between carbon dioxide and a $C_3$ compound formed from the three carbons of glycerol Pyruvic acid had been isolated from this fermentation (21) and was proposed as the $C_3$ compound

No real progress was made toward the identification of the actual components of this reaction until Krampitz and Werkman (22) discovered a heat-labile enzyme from *Micrococcus lysodeikticus* which catalyzed the decarboxylation of oxalacetate to pyruvate Later, with $C^{13}O_2$ it was possible to show that the reaction was reversible and that the enzyme could induce the incorporation of carbon dioxide into oxalacetate (17) The $C^{13}$ of the labeled carbon dioxide was found exclusively in the carboxyl adjacent to the methylene group of oxalacetate as predicted in Reaction 1 Since the only compounds added in this reaction were oxalacetate and carbon dioxide, and pyruvate and carbon dioxide were the only products formed, the evidence was fairly clear that the carbon dioxide was introduced into the oxalacetate with no alteration of the compounds other than perhaps minor transformations for activation such as to form phosphorylated compounds

With animals a similar type of direct evidence of fixation of carbon dioxide by oxalacetate β-carboxylase was not obtained although claims for proof of such a reaction were made on the basis of indirect results These claims have already been critically examined in a previous review (2)

Evans *et al* (23) in 1943 made an important contribution toward the proof that animal tissue fixes carbon dioxide by oxalacetate β-carboxylase, when they obtained a cell-free preparation from pigeon liver containing this enzyme The enzyme was heat-labile and catalyzed the decarboxylation of oxalacetate to pyruvate, just as the bacterial enzyme (22), but differed in one apparently minor respect in that it was activated only by $Mn^{++}$ instead of either $Mn^{++}$ or $Mg^{++}$ When both fumarate and pyruvate were substrates, the liver extract catalyzed a rapid fixation of carbon dioxide, as judged from the amount of $C^{11}$ in organic linkage when $C^{11}O_2$ was used as a tracer The net chemical change during this reaction consisted in a conversion of the fumarate to lactate and carbon dioxide The original pyruvate concentration remained unchanged during the reaction but very little $C^{11}O_2$ was fixed if the pyruvate was omitted from the reaction mixture

When pyruvate was the only added substrate and crude extracts were used there was considerable fixation of carbon dioxide, but with dialyzed or purified enzymes the $C^{11}$ fixed was reduced to a very small value The fixed $C^{11}$ in the latter case was almost quantitatively precipitated as dinitrophenylhydrazone (presumably of pyruvate) Evans *et al* (23) have considered this fixation of carbon dioxide with pyruvate to be an uncomplicated example of the primary fixation by Reaction 1 Since the amount of fixation was reduced by purification of the enzyme it is possible that necessary unknown reactants were removed by the procedures of purification This fixation may, thus, have the same character as the fixation with mixed substrates of pyruvate and fumarate in that it is not a simple reaction The results were, thus, not a very satisfactory demonstration of the primary reaction

Evans *et al* (23) proposed that the fixation with pigeon liver extract occurred by reversibility of the following reactions

(2) $C^{11}O_2 + CH_3\ CO\ COOH \leftrightharpoons C^{11}OOH\ CH_2\ CO\ COOH$

(3) $C^{11}OOH\ CH\ COH\ COOH \leftrightharpoons C^{11}OOH\ COH\ CH\ COOH$ (Shift of OH)

(4) $COOH\ CH\ CH\ COOH + H_2O \leftrightharpoons COOH\ CH_2\ CHOH\ COOH$

(5) $COOH\ CH_2\ CHOH\ COOH + CH_3\ CO\ COOH \leftrightharpoons COOH\ CH_2\ CO\ COOH + CH_3\ CHOH\ COOH$

Reactions 2 and 3 were proposed to account for the fixation when pyruvate was the only substrate and Reactions 2 through 5 when both pyruvate and fumarate were included Krampitz *et al* (17) have demonstrated that the shifting of the OH of enol-oxalacetate does not occur spontaneously via Reaction 3 They showed that excess $C^{13}$ in one carboxyl group of oxalacetate did not become spontaneously distributed in both carboxyl groups of oxalacetate and therefore the possibility of Reaction 3 occurring non-enzymatically was excluded

In order to further verify the reactions, Wood, in collaboration with Evans and Vennesland (24), studied the fixation in greater detail Carbon dioxide labeled with $C^{13}$ was used and the components of the reaction were degraded for determination of the location and concentration of the $C^{13}$ in each compound It was demonstrated that the $C^{13}$ was entirely in the carboxyl groups of the pyruvate, lactate, malate and fumarate and that the concentration of $C^{13}$ was approximately the same in all the carboxyl groups These results were, therefore, in complete agreement with the proposed Reactions 2, 4, and 5 of Evans *et al.* (23) However, an attempt was not successful to verify by direct methods that the liver oxalacetate $\beta$-carboxylase induced exchange of carbon dioxide in oxalacetate, in a manner similar to the bacterial preparation These studies indicated that the reversal of Reaction 1 was apparently too slow to account for the rapid exchange observed with fumarate and pyruvate There thus remained some doubt as to the mechanism of the primary reaction of the fixation by the pigeon liver extract

This uncertainty has largely been removed by the recent experiments of Utter and Wood (25) They have demonstrated that the decarboxylation of oxalace-

tate by the liver extract is reversible, when adenosine triphosphate (ATP) is added to the enzyme With $NaHC^{13}O_3$, oxalacetate, no ATP and the enzyme of liver extract a very slow exchange of $C^{13}$ in the oxalacetate was observed When ATP was added to the mixture the rate of fixation was comparable to that observed with pyruvate and fumarate Adenylic acid did not replace ATP in this reaction, nor did cocarboxylase $Mn^{++}$ is a necessary activator for the carboxylation and $Mg^{++}$ will not replace the function of the $Mn^{++}$, just as has been previously observed for the decarboxylation (23) Whether or not the requirement of ATP is a point of difference from the bacterial oxalacetate $\beta$-carboxylase is not apparent at present *It is possible that there is sufficient* ATP in the bacterial preparation to supply the requirements of the reaction

The function of the ATP in Reaction 1 is not evident It remains to be investigated whether ATP serves to phosphorylate oxalacetate or pyruvate and these esters are the active components of the reaction, or whether, for example, ATP acts in the synthesis of an essential phosphorylated coenzyme as Gunsalus *et al* (26) have observed with ATP and pyridoxal in their studies of tyrosine decarboxylase The function of the ATP is not to phosphorylate thiamin since cocarboxylase does not catalyze the exchange Neither is its function to phosphorylate pyruvate to give phosphopyruvate Phosphopyruvate will not replace the ATP function

It is interesting in this connection that Kalnitsky and Werkman (27) with an extract of *E coli* have observed the formation of oxalacetate or a chemically similar compound from pyruvate and bicarbonate under anaerobic conditions The oxalacetate was determined by the aniline citrate reaction and by the colorimetric method of Straub (28) However, Evans (1), on the basis of thermodynamic data, has calculated for the direct carboxylation of pyruvic acid, that the maximum amount of oxalacetate that could be formed would be only 0 06 per cent of the observed amount He has concluded that either the pyruvate forms some derivative of higher energy content which is the true substrate of carboxylation, or the bulk of the oxalacetate found was derived from another source Apparently Evans (1, p 281) misinterpreted Kalnitsky and Werkman's report, however, for although the latter authors speak of direct carboxylation they were not unaware that energy is necessary for the reaction They considered this aspect of the problem and suggested that the simultaneous formation of acetylphosphate from pyruvate by the *E coli* extract might be the logical source of energy for the endergonic reaction The finding by Utter and Wood that ATP is an essential component of the reaction gives some support to the contention that there may be a high energy derivative with which the actual carboxylation occurs

*$C_2$ and $C_1$ addition by the phosphoroclastic reaction* Early attempts to prove that there was fixation of carbon dioxide by $C_2$ and $C_1$ addition were not successful Krampitz *et al* (17) with $C^{13}O_2$ were unable to detect any reversibility in the oxidative decarboxylation of pyruvate with *Micrococcus lysodeikticus*, the $C^{13}$ was determined in the carboxyl of the pyruvate remaining after fifty per cent of it had been oxidized by the bacteria Evans (139) obtained similar negative

results with yeast carboxylase but Carson *et al* (29) reported a very small positive value. Because the pyruvate was separated as the 2,4-dinitrophenylhydrazone, which is not specific for this compound, and because the amount of $C^{11}$ fixed was so small the significance of the latter result is uncertain

Nevertheless, there was some evidence in certain bacterial fermentations that $C_3$ and $C_1$ combination did occur Slade *et al* (5) found that *Clostridium welchii* and *Cl. acetobutylicum* produced lactate in which $C^{13}O_2$ was fixed in the carboxyl group Since there was no detectable amount of $C_4$ dicarboxylic acids formed in these fermentations there was no evidence that the $C^{13}$ lactate arose *via* $C_3$ and $C_1$ fixation Also, Brown *et al* (30) could find no evidence that butyl alcohol bacteria dissimilated $C_4$ dicarboxylic acids This finding made still more remote the possibility that the $C^{13}$-lactate was formed from these acids

The above results were suggestive but were by no means conclusive The proof of $C_3$ and $C_1$ addition came from the study of a specific enzyme reaction, the phosphoroclastic split of pyruvate, which was first demonstrated by Utter and Werkman (31) with an extract from *E coli*

(6) $$CH_3\ CO\ COOH + H_3PO_4 \leftrightharpoons CH_3\ CO(OPO_3H_2) + HCOOH$$

The background of this discovery was as follows

Lipmann's important investigations of pyruvate oxidation with *Lactobacillus delbrückii* had pointed to acetyl phosphate as a key intermediate in metabolism (32), especially since the phosphate linkage in acetyl phosphate is of the energy-rich type (33) and is able to bring about the phosphorylation of adenylic acid Curiously, the phosphoroclastic reaction was first suggested as the result of a misunderstanding Silverman and Werkman (34) in an investigation of the conversion of pyruvate to acetyl-methylcarbinol by *Aerobacter*, presented evidence that phosphate was necessary for the reaction Lipmann (33, p 135) believing that they were working with the so-called hydroclastic split of pyruvate (Reaction 7)

(7) $$CH_3\ CO\ COOH + HOH \rightarrow CH_3\ COOH + HCOOH$$

and that phosphate had been found necessary for the reaction, suggested that the hydroclastic split might in reality be a phosphoroclastic split (Reaction 6)

Kalnitsky and Werkman (35) later found that cell free extracts of *E coli* were unable to dissimilate pyruvate after a short period of dialysis and that the system was reactivated by addition of phosphate The principal conversion was to acetic and formic acids, although succinate and lactate were also formed Utter and Werkman (31) then provided convincing proof of the phosphoroclastic reaction by showing that one mole of labile phosphate (assumed to be acetyl phosphate) arose for each mole of pyruvate converted to a $C_2$ compound by an *E coli* extract. Most of the acetyl phosphate was formed by the phosphoroclastic split but apparently about 15 per cent was formed by dismutation of the pyruvate to lactate and acetyl phosphate and carbon dioxide

In the phosphoroclastic cleavage of pyruvate the energy from the splitting is largely preserved in the acetyl phosphate The external energy loss from the

system is thus sufficiently low that the reaction becomes much more reversil than would be possible with the hydroclastic split (36) Utter *et al* (18) we able to demonstrate this reversibility by dissimilating pyruvate with the *E c* extract in the presence of $HC^{13}OOH$ The residual pyruvate was shown to co tain excess $C^{13}$ in the carboxyl group For example, after sixty minutes' anaerol incubation, thirty per cent of the pyruvate was broken down and the form contained 0 77 per cent excess $C^{13}$ and the carboxyl of the pyruvate 0 78 per ce Thus, the excess $C^{13}$ which had been added as formate was in complete equil rium with the pyruvate-carboxyl Since the carbon dioxide in the system at t conclusion of the experiment contained only 0 06 per cent excess $C^{13}$, it is cl that the heavy carbon pyruvate could not have resulted from oxalacetate carboxylase fixation of carbon dioxide, or any reaction involving carbon diox as such

When pyruvate was dissimilated in the presence of $CH_3C^{13}OOH$ and adenos triphosphate the residual pyruvate contained excess $C^{13}$ in the carbonyl positi This exchange was about twenty times slower than that with pyruvate and f mate, apparently because Reaction 8 was limiting

(8) $$CH_3\ C^{13}OOH + ATP \rightleftharpoons CH_3\ C^{13}O(OPO_3H_2) + ADP$$

(9) $$CH_3\ C^{13}O(OPO_3H_2) + HCOOH \rightleftharpoons CH_3\ C^{13}O\ COOH + H_3PO_4$$

The reversibility of Reaction 8 has been shown by Lipmann (37)

Although the proof that fixation occurs as pictured in Reaction 6 is quite co clusive, perhaps one final piece of evidence is desirable to show that it is ace phosphate rather than another derivative of pyruvate which is the compone of the rapid equilibrium with formate This proof would involve determini whether or not heavy carbon acetyl phosphate equilibrates with pyruvate *rapidly as labeled formate*

Lipmann and Tuttle (16) have extended the investigations of the equilibri of the phosphoroclastic reaction using acetyl phosphate and formate and det mining chemically the pyruvate in equilibrium mixtures The equilibrium far toward the formation of acetyl phosphate and formate so that a very sm amount of pyruvate is formed However, considered in view of proof provid by the heavy carbon experiments it seems quite probable that the measured co pound is really pyruvate According to these measurements the equilibri constant is roughly $10^{-2}$ for the condensation reaction

The exchange of radioactive inorganic phosphate with the phosphorus of ace phosphate was also determined The equilibrium between the organic and i organic phosphate was reached in about one-half hour Lipmann and Tut have concluded that the phosphate exchange is caused predominantly by F action 6 although the possibility of other reactions occurring is considered It to be noted that the rate of exchange was not influenced by addition of any r actant other than acetyl phosphate This is surprising if the exchange occurs the phosphoroclastic reaction

Closely related to the above investigations are the experiments by Koepsell

and Johnson (38) and Koepsell *et al* (39) They obtained a cell free extract from *Clostridium butylicum*, which catalyzed the fermentation of pyruvate to acetate, carbon dioxide and hydrogen The amount of hydrogen evolution during the reaction was proportional to the concentration of inorganic phosphate, but with ordinary methods for phosphate determination no phosphate ester could be detected (38) However, by using a procedure applicable to the determination of labile phosphate esters, an ester could be separated which quite certainly was acetyl phosphate in mixture with butyryl phosphate (39) The butyryl phosphate apparently is formed by transfer of the phosphate of acetyl phosphate to butyric acid

Reaction 10 which is catalyzed by the extract of the butyl bacteria is thus almost identical with that by the extract from *E coli*

(10) $CH_3\ CO\ COOH + H_3PO_4 \rightleftharpoons CH_3\ CO(OPO_3H_2) + CO_2 + H_2$

The difference in the reactions is that formate is not a product and apparently not an intermediate since formate is not broken down by the enzyme from butyl bacteria Lipmann and Tuttle (16) have provided evidence for the view that Reaction 10 is reversible They showed that about the same amount of pyruvate is formed by this enzyme from acetyl phosphate and carbon dioxide and hydrogen as was observed with the extract from *E coli* and acetyl phosphate and formate Very recently Wilson, Krampitz and Werkman (40) have added to the evidence by experiments with $C^{13}O_2$ Using a cell free extract of *Cl butylicum* with pyruvate, $C^{13}O_2$ was found to exchange rapidly with the carboxyl group of pyruvate It is thus clear that carbon dioxide may be fixed by $C_2$ and $C_1$ addition by two different bacteria (intact *E coli* cells can reduce carbon dioxide with hydrogen to formate) Furthermore, it is reasonably certain that acetyl phosphate is an intermediate in the reaction

Whether or not acetyl phosphate plays a rôle in animal tissue is not known at present The comparative biochemistry of different tissues has thus far proved similarity to be the rule and it seems probable that animal tissue will be found to produce acetyl phosphate Lipmann (41) has recently described an enzyme occurring in skeletal muscle which rapidly and specifically dephosphorylates acetyl phosphate Its presence in tissue might readily interfere with the detection of any acetyl phosphate which was present The establishment of acetyl phosphate as an intermediate in animal metabolism will not necessarily imply that animals can fix carbon dioxide by $C_2$ and $C_1$ condensation It is to be noted that there are no reactions known to occur in animals which are comparable to the reactions which are reversible in bacteria, i e , reactions in which formate or hydrogen gas is produced If the dismutation of pyruvate to lactate and acetate and carbon dioxide were found to be reversible, the possibility of fixation of carbon dioxide by $C_2$ and $C_1$ addition in animals would seem much more probable, since this conversion apparently occurs in animal tissue (42, p 210)

Although carbon dioxide fixation in the carboxyl group of lactic acid, as shown by Wood *et al* (11), (24) with pigeon liver and with the liver extract may possibly occur by $C_2$ and $C_1$ condensation, the fixation seems to be adequately accounted

for by $C_3$ and $C_1$ condensation The liver extract apparently does not break down pyruvate, therefore the possibility of $C_2$ and $C_1$ addition in this case seems remote

*$C_4$ and $C_1$ addition by oxalosuccinate carboxylase* Ochoa and Weisz-Tabori (43) have recently made the very interesting discovery that crude solutions of isocitric dehydrogenase prepared from heart muscle contain an enzyme which catalyzes Reaction 11

(11) $COOH\ CH_2\ \underset{\displaystyle COOH}{\overset{\displaystyle |}{CH}}\ CO\ COOH \rightleftharpoons$

Oxalosuccinic acid

$$COOH\ CH_2\ CH_2\ CO\ COOH + CO_2$$

$\alpha$-Ketoglutaric acid

Chemical synthesis of oxalosuccinic acid was accomplished for the first time and the decarboxylation of the acid was then studied with the enzyme preparation The enzyme is thermolabile and is activated by $Mn^{++}$ but not by $Mg^{++}$ It can be precipitated with acetone from aqueous solution without loss of activity and is almost completely precipitated by acidification to pH 5 2 The enzyme does not catalyze the decarboxylation of oxalacetic acid

Previously the decarboxylation of oxalosuccinate had been considered to be a spontaneous, non-enzymatic reaction (44) However, Evans (1) had suggested that the decarboxylation might be enzymatic, cf Moulder *et al* (138) for the complete account of the investigation This suggestion was based on observations made with pigeon liver extract in studies of Reaction 12

(12) pyruvate + citrate $\rightarrow$ $\alpha$-ketoglutarate + carbon dioxide + lactate

While the pigeon liver extract catalyzed this reaction, similar extracts from pigeon breast muscle did not, although the requisite enzymes, lactic and isocitric dehydrogenases together with coenzymes, were present However, when a small amount of purified pigeon liver extract was added to the muscle system the reaction proceeded Moulder *et al* suggest that the liver preparation might serve in the decarboxylation of oxalosuccinate, and that this would explain the necessity of $Mn^{++}$ or $Mg^{++}$ ions for isocitrate oxidation These ions were considered unusual cofactors for the pyridine nucleotide enzymes, lactic and isocitric dehydrogenases

These authors (138) also pointed out the interesting fact that Reaction 12 involves the interaction of a lactate-pyruvate system which is thought to be coenzyme I specific with an isocitrate-$\alpha$-ketoglutarate system which is thought to be coenzyme II specific They raise the question of whether or not these enzymes may not react with both cofactors

Not only has a specific enzyme for oxalosuccinate been demonstrated (43) but Ochoa (19) has shown in addition that the reaction is reversible, i e , that carbon dioxide combines with $\alpha$-ketoglutarate to form oxalosuccinate Since labeled carbon dioxide was not used it was necessary to remove the oxalosuccinate by

reduction to isocitrate (Reaction 13), in order to shift the reaction toward carbon dioxide fixation Reaction 13 was followed by determining spectrometrically the $TPN_{ox}$[2]

(13) $COOH\ CH_2\ CH(COOH)\ CO\ COOH + TPN_{red} \rightleftharpoons$

Oxalosuccinic acid

$COOH\ CH_2\ CH(COOH)\ CHOH\ COOH + TPN_{ox}$

Isocitric acid

In the presence of the heart enzyme the oxalosuccinate for Reaction 13 can be replaced by α-ketoglutarate and carbon dioxide The α-ketoglutarate and car bon dioxide combine according to Reaction 11 and the resulting oxalosuccinate is reduced to isocitrate The reaction can be shifted still further toward carbon dioxide fixation by combining it with a glucose-6-phosphate dehydrogenase system. $TPN_{red}$ is supplied by the glucose-6-phosphate dehydrogenase system (Reaction 14) for reduction of the oxalosuccinate

(14) glucose-6-phosphate + $TPN_{ox.} \rightarrow$ 6 phosphogluconate + $TPN_{red}$

The net result of the combined systems is the following dismutation

(14a) α-ketoglutarate + $CO_2$ + glucose-6-phosphate ⇌
isocitrate + 6 phosphogluconate

The isocitrate was determined with isocitric dehydrogenase

The evidence is thus clear that carbon dioxide combines with α ketoglutarate to form oxalosuccinate Since oxalosuccinate and oxalacetate are both β keto acids and the carboxylation in both cases occurs β to the keto group, the reactions have much in common It is possible that further study may reveal that ATP is a component of the oxalosuccinate carboxylase reaction, as it is in the oxalace tate carboxylase reaction It is to be noted that both reactions are simple carboxylations whereas the reaction of acetyl phosphate with carbon dioxide involves a simultaneous reduction

Ochoa has pointed out that the equilibrium of the isocitrate system in the presence of aconitase would be shifted toward carboxylation of α ketoglutarate, because over ninety per cent of the isocitric acid would be removed to form cis aconitate and citrate The aconitase isocitric dehydrogenase-oxalosuccinate carboxylase system could then play an important part in the biological utilization of carbon dioxide

In the Krebs cycle it is usual to consider the reactions as oxidative processes in which the $DPN_{red}$ is kept at a low concentration and the reactions are shifted toward decarboxylation It is evident, however, since the reactions are revers ible, that under suitable conditions the reactions may be shifted toward syn

[2] TPN = triphosphopyridine nucleotide.

thesis These reactions may then serve in synthesis to supply needed components for maintenance and biochemical reactions of the animal Furthermore, the reactions we have just considered pass in steps from $C_2$ (acetate), to $C_3$ (pyruvate) to $C_4$ (oxalacetate), then miss a step $C_4$ to $C_5$ and then go from $C_5$ ($\alpha$-ketogluta rate), to $C_6$ (isocitrate) If the conversion of succinate to $\alpha$-ketoglutarate were shown to be reversible, a model would then be available for reversal of the Krebs cycle, in which isocitrate would be synthesized from a $C_2$ compound and 4 carbon dioxide molecules It is clear that reactions gradually are being derived from studies with animals and heterotrophic bacteria, which may eventually help to explain the chemistry of photosynthesis

SUMMARY Three initial reactions for the fixation of carbon dioxide have been demonstrated

Carboxylation by the oxalacetate $\beta$-carboxylase reaction was first demon strated with bacteria and has now been shown conclusively with Evans' liver preparation ATP is an essential component of the reaction It is probable that a high energy derivative of oxalacetate or pyruvate is formed with which the carboxylation occurs

Fixation of carbon dioxide by $C_2$ and $C_1$ addition has been demonstrated in bacteria The $C_2$ component is almost certainly acetyl phosphate, which con denses with formic acid or carbon dioxide and hydrogen A corresponding reaction in animal tissue has not been demonstrated as yet

The carboxylation of $\alpha$-ketoglutarate to yield oxalosuccinate has recently been demonstrated with a specific enzyme from heart muscle There is some evidence of a similar enzyme in liver

Except for one carboxylation, the synthesis of a $C_6$ compound from acetic acid and four molecules of carbon dioxide can now be pictured with reversible enzyme reactions It is obvious that these studies may help to explain the chemistry of photosynthesis

SECTION II MECHANISM OF FIXATION OF CARBON DIOXIDE IN DIFFERENT COMPOUNDS Fixation of carbon dioxide has been shown to occur in some 17 different compounds and probably occurs in others For a number of these compounds the mechanism of fixation has not been clarified but there is no reason to believe the reactions may not be of considerable biochemical importance. For others there is a fairly clear concept of the reactions which, for the most part, are largely based on the same fundamental mechanisms This subject has been considered in a previous review (2) and will be summarized in detail in this com- munication only in relation to the more recent developments The compounds will be considered in the order of increasing length of the carbon chain and since there have been several recent investigations on the fixation of carbon dioxide in acetate and glycogen, these compounds will be discussed in some detail

*$C_1$ compounds* *Methane* The reduction of carbon dioxide to methane has been demonstrated with labeled carbon by Barker *et al* (45) with methane bacteria According to Barker's views (46), all types of methane fermentations by bacteria may be considered as oxidations in which carbon dioxide acts as the hydrogen acceptor

(15) $$4H_2A + CO_2 \rightarrow 4A + 2H_2O + CH_4$$

$H_2A$ is an oxidizable molecule and A is the oxidation product

The intermediate compounds of the reduction of carbon dioxide to methane are not known (Barker (47))

*Urea* The synthesis of urea by the ornithine cycle as proposed by Krebs and Henseleit (48) involves utilization of carbon dioxide Proof that carbon dioxide is fixed in urea by liver has been shown with $C^{13}O_2$ (49) and $C^{11}O_2$ (50)

*Formic acid* Woods in 1936 (51) demonstrated that carbon dioxide can be reduced by gaseous hydrogen to formic acid with *E coli* This reaction, which is catalyzed by hydrogenlyase, probably occurs with other bacteria In fermentations of glucose by *Aerobacter indologenes* (5) and by *Cl butylicum* (30) and in the dissimilation of pyruvate by *E coli* (52), (18), $C^{13}O_2$ has been shown to be converted to formate Since formate can be produced from pyruvate directly without exchange of carbon dioxide with the formate (53) it is possible in some of these fermentations that the $C^{13}$ formate was formed from intermediary $C^{13}$ pyruvate However, the above named organisms contain hydrogenlyase and the $C^{13}$ formate sometimes contained more $C^{13}$ than the carboxyls of the pyruvate or lactate, so it is reasonably certain that some of the $C^{13}$ formate arose by direct reduction

*$C_3$ compounds Acetic acid* Carbon dioxide is fixed in the carboxyl group of acetic acid arising in glucose fermentations by *Aerobacter indologenes* and by *Cl welchii* (5) A different type of fixation occurs in the fermentations of uric acid by *Cl. acidi urici* (6) In this case both positions of the produced acetic acid contained fixed carbon and sixty-seven per cent of the radio-activity was in the methyl group and thirty three per cent in the carboxyl group

Another organism which converts carbon dioxide to acetic acid is *Cl thermoaceticum* It ferments glucose (54) and pyruvate or xylose (55) with the formation of more than two moles of acetate per mole of carbohydrate and more than one mole per mole of pyruvate This is more acetate than can arise by the ordinary $C_2$ and $C_1$ type of cleavage through which fermentations have generally been considered to occur Barker and Kamen (134) have very recently demonstrated with this organism that carbon dioxide is fixed in both positions of acetate, when glucose is fermented in the presence of $C^{14}$ $Na_2CO_3$ The glucose was found quantitatively converted to acetate, $C_6H_{12}O_6 = 3CH_3COOH$ The authors present evidence that the glucose is dissimilated as follows

$$C_6H_{12}O_6 + 2H_2O = 2CH_3\,COOH + 2CO_2 + 8H$$

and that the resulting $CO_2$ is reduced to acetate

$$8H + 2CO_2 = CH_3COOH + 2H_2O$$

Barker *et al* (135) have also found that *Butyribacterium rettgeri* fixes carbon dioxide during the fermentation of lactic acid to acetic and butyric acids In the presence of $C^{14}O_2$ both carbons of the acetic acid contained isotope as well as all carbons of the butyric acid It is suggested that acetic acid is formed by condensation of carbon dioxide and evidence is presented that the butyric acid is

formed by condensation of acetic acid These fixations which occur in more than one position of the acetate involve apparently, though not necessarily, a carbon to carbon linkage in which both components are formed from carbon dioxide It is possible that part of the acetate molecules might have fixation in the methyl group alone and another part of the molecules in the carboxyl group The analysis would not detect the difference between this mixture and a material in which both the carbons of acetate contained isotope · However, Weiringa (136) has shown that another species of Clostridium, *Cl aceticum*, can reduce carbon dioxide to acetate with hydrogen gas in a medium containing an extract of mud. In this case, apparently both carbons in the acetate are formed from inorganic carbon It therefore seems reasonable that other species of Clostridium may bring about such a synthesis

A mechanism for these fixations has not been suggested but they are of special interest because of the similarity to autotrophic synthesis The mechanism may be by a cycle involving repeated addition of carbon dioxide to an organic substance, following which acetate is split off This cycle may involve some of the primary fixation reactions described in Section I, for example, a reversal of the tricarboxylic acid cycle could give such an acetate

Slade and Werkman (56) have studied in some detail the mechanism of formation of acetate by *Aerobacter* in which carbon is fixed exclusively in the carboxyl group They suggest that acetate may be formed from succinate by *Aerobacter* according to Reaction 16 The actual components of the reaction may be derivatives of acetate and succinate

(16) $$C^{13}OOH\ CH_2\ CH_2\ C^{13}OOH + 2H \rightleftharpoons 2CH_3\ C^{13}OOH$$

Evidence of Reaction 16 was obtained by adding $CH_3\ C^{13}OOH$, $C^{13}H_3\ C^{13}OOH$, and $C^{13}OOH\ CH_2\ CH_2\ C^{13}OOH$ to glucose fermentations The succinate formed in the fermentations with $CH_3\ C^{13}OOH$ contained $C^{13}$ exclusively in the carboxyl groups while that formed with $C^{13}H_3\ C^{13}OOH$ contained equal concentrations of $C^{13}$ in the methylene and carboxyl carbons From the succinate, acetate with the $C^{13}$ only in the carboxyl group was formed

These conversions could not have resulted from carbon dioxide fixation after oxidation of the $C^{13}$-compounds to carbon dioxide, first, because the $C^{13}$ concentration of the carbon dioxide was lower than that in the acetate or succinate which was formed, and second, because by carbon dioxide fixation the $C^{13}$ would only be in the carboxyl group (5) whereas, when doubly labeled acetate was used, $C^{13}$ was found to occur equally in all positions of the succinate It also is apparent that the $CH_3C^{13}OOH$ was not converted extensively to acetyl phosphate and then to pyruvate by the phosphoroclastic reaction, for in such a case it would be expected that succinate with $C^{13}$ in the methylene groups would have resulted by the oxalacetate $\beta$-carboxylase fixation and reduction of the resulting oxalacetate

(17) $$CH_3\ C^{13}O(OPO_3H_2) + HCOOH \rightleftharpoons CH_3\ C^{13}O\ COOH + H_3PO_4$$

(18) $$CO_2 + CH_3\ C^{13}O\ COOH \rightleftharpoons COOH\ CH_2\ C^{13}O\ COOH \rightleftharpoons COOH\ CH_2\ C^{13}H_2\ COOH$$

Kalnitsky *et al* (53) have obtained somewhat similar results with a cell free enzyme preparation from *E coli* When pyruvate was fermented by this preparation in the presence of $CH_3C^{13}OOH$, succinate was formed which contained $C^{13}$ *in the carboxyl groups* Since there was no excess $C^{13}$ *in the carbon dioxide* produced in the dissimilation it is clear that the $C^{13}$-succinate was not formed by fixation of carbon dioxide It appears that this enzyme preparation offers a *promising opportunity to study this interesting reaction*

Slade and Werkman consider their results to constitute conclusive proof for Reaction 16 While their evidence for Reaction 16 is quite convincing, it never theless is possible that the changes observed result by some other mechanism. For example, in the tricarboxylic acid cycle (fig. 1, p 212) succinate with labeled carbon in the carboxyl group might be formed from carboxy labeled acetate by Steps[1] 19, 5, 6, 7, 8, 10 Also from the doubly labeled acetate, succinate with the isotope in both the methylene and carboxyl groups would result In addition, by the reversal of these steps labeled acetate could be formed from succinate At the present time there is no evidence for the reversibility of Step 10 of the cycle, i.e., succinate to α-ketoglutarate, but likewise there is no direct evidence with an isolated enzyme system plus succinate and acetate that Reaction 16 as such is reversible

The problem is of considerable importance, for it raises again the question of whether or not oxidative conversion of pyruvate to carbon dioxide may occur according to the older proposal of Thunberg (57) This proposal has for some time been dropped from consideration largely because of the lack of evidence for Reaction 16 This cycle as shown below starts with two molecules of pyruvate, one is again regenerated and three molecules of carbon dioxide are formed

$$\begin{array}{ccc} 2CH_3\ CO\ COOH & \longrightarrow & 2CH_3\ COOH + 2CO_2 \\ \uparrow & & \downarrow \\ \uparrow\!\!-CO_2 & & \downarrow \\ COOH\ CH_2\ CO\ COOH & \longleftarrow & COOH\ CH_2\ CH_2\ COOH \end{array}$$

With animal tissue there has been no evidence of fixation of carbon dioxide in acetate and as yet no comparable proof for Reaction 16

The earlier results of Sonderhoff and Thomas (108) should be considered in this connection These results were obtained by isolating succinate and citrate from the products of the oxidation of $CD_3$ COOH by yeast The concentration of the deuterium in the succinate and citrate was then determined Since the four methylene hydrogens of the six hydrogen linkages of succinate are stable and not subject to exchange with hydrogen, the ratio of deuterium to hydrogen plus deuterium would be expected to be 0 67 (4/6), if succinate was formed by direct condensation of $CD_3$ COOH. Likewise for citrate in which four out of eight of

[1] The individual conversions of figure 1 have been numbered and are referred to as *Step* 1, etc on the other hand the conversions which have been illustrated in the text proper are designated as *Reaction* 1, etc

the hydrogens are stable, this ratio would be 0 50 The observed ratios were 0 27 (40 per cent of the possible 4D) for succinate and 0 28 (56 per cent of the possible 4D) for citrate On the assumption that there was some dilution of the succinate by endogenous reactions it seemed probable that approximately 2D from the acetate were incorporated in the succinate and citrate

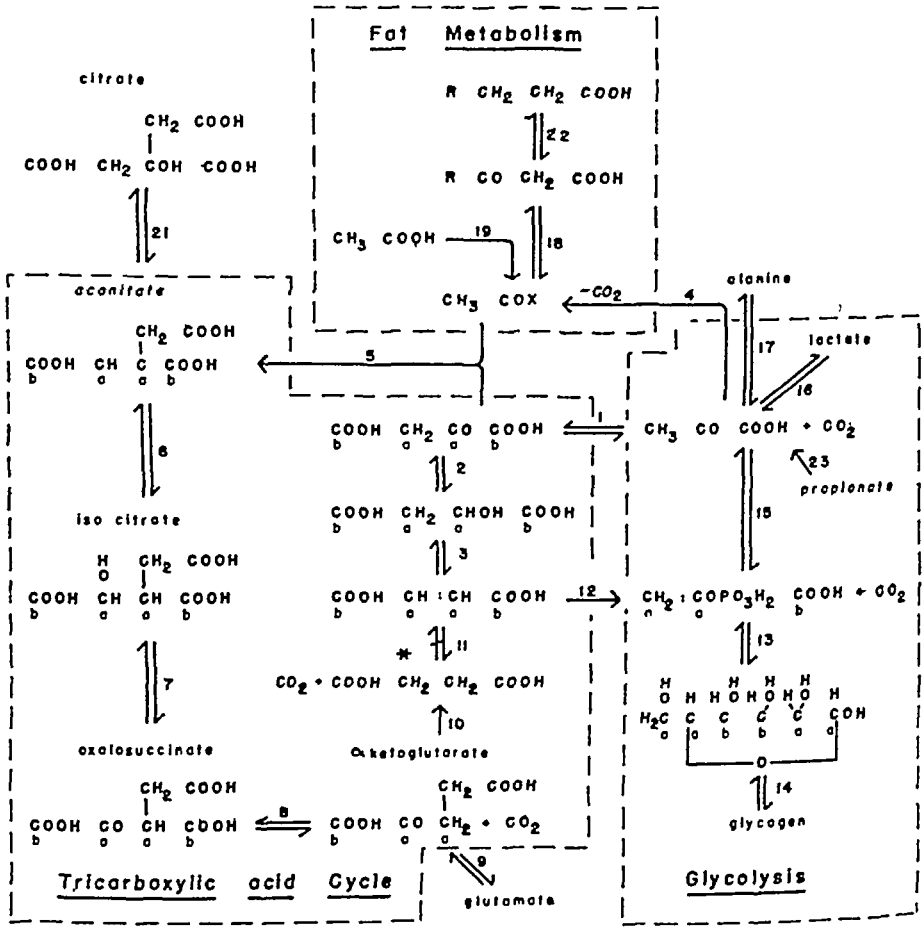



Fig 1 Interrelationships of the tricarboxylic acid cycle and carbohydrate, fat, and protein metabolism

* Malonate inhibition

Lynen (87) has pointed out that the succinate might be formed via the tricarboxylic acid cycle, figure 1 However, he concludes that the symmetrical citrate molecule could not be an intermediate for if it were, the succinate would contain only 1D His point is clear if one traces the hydrogen atoms on the methyl of the acetyl compound of figure 1 through Steps 5, 6, 7, 8, 10 In all of these steps the hydrogen of the original methyl group is stable and the result-

ing succinate would therefore contain 2D However, if a direct conversion of oxalacetate and acetate occurred to give citrate with the following formula

$$\begin{array}{l} \qquad\quad CD_2\ COOH \\ \qquad\quad\ \ | \\ COOH\ CH_2\ COD\ COOH \end{array}$$

then upon conversion of citrate to aconitate (Step 21) the deuterium on the carbinol group would be lost Furthermore, the deuterium labeled methylene group and normal methylene group would be converted equally to the carbonyl in oxalosuccinate Thus, one half of the deuterium of the methylene groups of citrate would be lost, therefore, there would be only 1D in the succinate formed from the citrate

There is one point, however, that is not accounted for in these considerations, i.e., how there can be a net production of citrate and succinate via the cycle with acetate as the only substrate The tricarboxylic acid cycle does not provide for this fact In the cycle a $C_4$ dicarboxylic acid is required for the original condensation (Step 5) and only by the completion of the cycle is the $C_4$ acid regenerated It thus becomes necessary to assume that enough $C_4$ dicarboxylic acids were produced in Sonderhoff and Thomas' experiments to provide for the observed accumulation of citrate and succinate It seems questionable whether the endogenous respiration could be the source of this quantity of $C_4$ acids

Actually the experimental results may be consistent with a mechanism involving formation of succinate by direct condensation of acetate via Reaction 16 The succinate formed from $CD_3$ COOH by this reaction would contain four D but the ultimate concentration of D in the succinate of the reaction mixture would depend on the relative rates of several reactions For example, by reversible conversion of succinate to fumarate, Step 11, the D from the succinate could be rapidly lost Also succinate formed via Step 10 would influence the D content of the succinate It is clear that a reliable prediction of the D content of the succinate cannot be made unless the relative rates of the reactions are known

Furthermore, in the conversion of succinate to oxalacetate by Steps 11, 3, and 2, three of the hydrogens of the methylene groups of the succinate are removed and in addition the fourth hydrogen would in part be lost via formation of the enol of oxalacetate It is thus evident that even though the $C_4$ dicarboxylic acid were formed from trideuteroacetate by direct condensation, the citrate formed via Steps 5 and 21 would not be expected to contain much more than 2D It is apparent therefore that the results of Sonderhoff and Thomas (108) are not in disagreement with formation of succinate by direct condensation of acetate Obviously deuterio labeled acetate is not a satisfactory compound for study of this particular problem Further investigation is needed, before the reactions can be defined by which yeast forms citrate and succinate from acetate

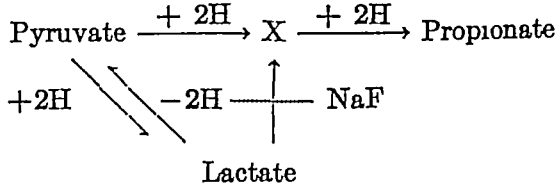
*$C_3$ compounds* *Pyruvic acid* The fixation of carbon dioxide in pyruvate has been demonstrated with *E coli* (18) *Cl butylicum* (40), and with pigeon liver extract (24) The mechanism of these fixations is by the phosphoroclastic re

action with the bacterial preparations and probably by oxalacetate $\beta$-carboxylase with pigeon liver, cf Section I, p 205

*Lactic acid* Carbon dioxide fixation in lactate has been demonstrated with a large number of species of bacteria (5), (30) and with pigeon liver (11), (24) The lactate presumably is derived from pyruvate in which fixation occurs by the two mechanisms mentioned above

*Propionic acid* Carbon dioxide is fixed in propionate during fermentations of glycerol, glucose or pyruvate by propionic acid bacteria (58), (52) The fixed carbon is exclusively in the carboxyl group of the propionate (59), (60) The mechanism of propionate formation is not known but from a consideration of the amount of carbon dioxide fixed in the propionate and succinate, it seems probable that the propionate is derived from a symmetrical dicarboxylic acid in which only one of the carboxyls is from fixed carbon dioxide This derivation accounts for the fact that in glycerol fermentations the propionate carboxyl contains roughly one-half as much excess $C^{13}$ as the carbon dioxide and the same per cent as the average of the succinate carboxyls (52), (2) It is necessary in this scheme to assume that the rates of the reactions are rapid toward succinate and propionate, otherwise by reversal of the reactions both carboxyls of the symmetrical acid would be in equilibrium with the $C^{13}O_2$

Related to this problem are the results of Barker and Lipmann (61) with the propionic acid bacteria They found, in confirmation of Chaix-Audermard (62), that sodium fluoride inhibits the conversion of lactate to propionate but not of pyruvate to propionate In studying the details of the inhibition it was found that the reduction of lactate but not the oxidation of lactate was inhibited by the fluoride Moreover, lactate did not accumulate in the fermentation of pyruvate in the presence of fluoride This observation was considered as evidence that lactate is not an intermediate in the reduction of pyruvate to propionate The following scheme was suggested by Barker and Lipmann to account for their observations




The hypothetical intermediate compound X is not believed to be acrylic acid It is possible, in view of the $C^{13}$ experiments, that X is a dicarboxylic acid which is formed by the fixation reaction

*Propyl alcohol* Propyl alcohol containing fixed carbon is formed in the fermentation of glycerol (52) It seems likely that it is formed by reduction of propionate which contains fixed carbon

*$C_4$ compounds* *Oxalacetic acid* The fixation of carbon dioxide in oxalacetate has been shown conclusively by Krampitz *et al* (17) with bacteria and by Utter

and Wood (25) with pigeon liver extract The reaction is catalyzed by oxalacetate β-carboxylase and is considered in detail in Section I, p 200

*Succinic acid* Bacteria (58), (52), (5), (53), protozoa (9) and pigeon liver (11) have been shown with labeled carbon to fix carbon dioxide in succinate during the dissimilation of a variety of substrates It is interesting that the fixation studied by Kalnitsky *et al* (53) was obtained with a cell free preparation Prior to the above investigation with protozoa (9) Searle and Reiner (8) had shown that the protozoan, *Trypanosoma lewisi*, could cause a net uptake of carbon dioxide in glucose dissimulations Labeled carbon dioxide was not used in the investigation, therefore the fixed carbon could not be located but it very likely was fixed in succinate, since the carbon dioxide assimilated was equivalent to the succinate formed

All investigators have concluded that the fixation of carbon dioxide in succinate is by the oxalacetate carboxylase Reaction 1 and by subsequent reduction of the oxalacetate In every case of fixed carbon dioxide in succinate the fixed carbon has been found to be exclusively in the carboxyl groups. It perhaps should be mentioned that in view of Slade and Werkman's (56) observation that acetic acid may condense to succinate (Reaction 16), it is conceivable that carbon dioxide could be fixed in acetate and then converted to succinate Succinate with fixed carbon might be formed in this case independent of $C_3$ and $C_4$ addition

*Fumaric and malic acids* Fumarate and malate are considered intermediates in the conversion of oxalacetate to succinate and it is to be expected that these compounds would contain fixed carbon Nishina *et al* (63) with radioactive carbon have demonstrated the synthesis of malate and fumarate from pyruvate and carbon dioxide in fermentations by *E coli* Also, Foster *et al* (7) with the mold *Rhizopus nigricans* have shown that carbon dioxide is fixed exclusively in the carboxyl groups of fumarate Similarly, in pyruvate dissimilations by pigeon liver (11), (24), the fixed carbon is in the carboxyl groups of the malate and fumarate

*Butyric acid* Carbon dioxide is fixed in butyrate by *Butyribacterium rettgeri* (135) (cf acetate above)

*$C_4$ compounds α-Ketoglutaric acid* The first proof of carbon dioxide fixation in a carbon to carbon linkage by animal tissue was Evans and Slotin's (64) demonstration that pigeon liver when acting on pyruvate synthesizes α-ketoglutarate containing carbon from $C^{11}O_2$ It was shown by Wood *et al.* (65), (11) and independently by Evans and Slotin (66) that the fixed carbon was exclusively in the carboxyl alpha to the keto groups of this dicarboxylic acid. The statement is incorrect (67) that Evans and Slotin proved this location of the fixed carbon in their first paper (64), no discussion concerning the location of the fixed carbon or of a degradation of the α-ketoglutarate occurs in this report

Thus far there has been no proof of fixation of carbon dioxide in α-ketoglutarate by molds, yeast, protozoa or bacteria, probably because the compound is difficult to isolate in dissimilations by these organisms

The mechanism of fixation of carbon dioxide in α ketoglutarate will be considered in Section III, p 221, under the tricarboxylic acid cycle

*$C_6$ compounds* *Oxalosuccinic acid* At present there is but one report (1 concerning fixation in this compound and it has been considered in detail Section I

*Citric acid* Neither isocitrate nor citrate has been isolated as yet from pige liver experiments in which labeled carbon dioxide was used Thus, there is direct proof of fixation in these compounds by pigeon liver, though such is to expected according to the tricarboxylic acid cycle In the experiments of Och (19) with heart muscle preparation, $\alpha$-ketoglutarate was converted to isocitra and the isocitrate therefore contained fixed carbon This experiment was do with non-labeled carbon dioxide

The only direct proof of fixation of carbon dioxide in citrate has been provid by Foster *et al* (7) with the mold *Aspergillus niger* They have shown th citric acid as formed in the fermentation of sucrose contains fixed carbon whi apparently is restricted to the carboxyl groups It is suggested that the mech nism may be that of the Krebs cycle

*Glycogen* The group of investigators at Harvard has contributed very i teresting information on the fixation of carbon dioxide (12), (68), (69) and t conversion of lactate (70), (71) and lower fatty acids (72) (acetate, propionat butyrate) to glycogen These experiments are especially significant becau they were the first in which the *in vivo* fixation of carbon dioxide was studi with normal animals Likewise, the experiments of Lorber *et al* (13) with t isolated working heart give evidence that fixation of carbon dioxide in glycog is a normal process in this organ

The procedure followed in the Harvard fixation experiments was to fast t rats for 24 hours, then to feed 150 mgm of lactate (12) or 300 to 600 mgm glucose (68) by stomach tube and to administer radioactive bicarbonate intr peritoneally in five doses at half hour intervals The glycogen was isolat from the liver at the end of the 2 5 hour period

The results were quite similar after either lactate or glucose feeding Th glycogen contained 0 3 to 1 5 per cent of the administered radioactive $C^{11}$ a 45 to 75 per cent of the $C^{11}$ was eliminated in the expired air An average of per cent of the liver glycogen was calculated to have been derived from fix carbon dioxide with lactate and 13 per cent with glucose In the muscle glycog after lactate feeding there was no significant incorporation of $C^{11}$ With gluco there was an appreciable amount, the radioactivity on comparable quantities glycogen being 10 to 30 per cent of that of the liver glycogen

The mechanism proposed by Solomon *et al* (12) for the conversion of pyruvat lactate, etc , to glycogen has been of special interest because it assigned to th fixation reaction an essential function in glycogen synthesis This function wa to circumvent the only step believed irreversible in glycolysis, i e , the conversi of pyruvate to phosphopyruvate (73) This reaction has now been shown to b reversible by Lardy and Ziegler (74) so that the fixation reaction can no long be considered obligatory on this basis It is still conceivable, of course, th the fixation may in part at least supply the phosphopyruvate for the synthesis

The mechanism proposed by Solomon *et al* for the fixation of carbon dioxide in glycogen was as follows

(19) $C^*O_2 + CH_2\ CO\ COOH \leftrightharpoons C^*OOH\ CH_2\ CO\ COOH$

(20) $C^*OOH\ CH_2\ CO\ COOH \leftrightharpoons C^*OOH\ CH\ CH\ COOH$

(21) $C^*OOH\ CH\ CH\ COOH \rightarrow CH_2\ C(OPO_3H_2)\ C^*OOH + CO_2$

or

$CH_2\ C(OPO_3H_2)\ COOH + C^*O_2$

The net result of these reactions is that pyruvate is phosphorylated and carbon dioxide is fixed in the carboxyl of the phosphopyruvate The remainder of the path to glycogen was viewed as the reversal of the current schemes of glycolysis by which two molecules of pyruvate are converted to a molecule of glucose In this conversion the carboxyls of the pyruvate make up the 3 and 4 positions of the glucose The Harvard group considered that one out of two of these carbons, on the average, would contain fixed carbon The fixed carbon in the glycogen, thus, should be 16 7 per cent (one in six) This value was approached in the Harvard experiments, on the basis of calculations which included certain assumptions The finding of approximately this amount of fixed carbon in the glycogen was considered the principal evidence for the scheme However, if the rates of Reactions 19 and 20 were sufficiently rapid to the left both carboxyls of fumarate would be in equilibrium with the carbon dioxide and in this case fixed carbon would be in all molecules of the pyruvate The fixed carbon in the glycogen would then be 33.3 per cent It seems possible that the observation of approximately 16 per cent fixation may have resulted from fortuitous circumstances

Wood *et al.* (75) have recently conducted some further studies on the fixation of carbon dioxide in glycogen. They determined the position of the fixed carbon in the glucose unit of the glycogen. The general procedure of treatment of the animals was similar to that of the Harvard experiments, except that $C^{13}$-bicarbonate was used The liver glycogen was isolated and then converted to glucose which was then degraded so that the position of the heavy carbon in the glucose could be determined

Two different types of degradation were used In the first the glucose was fermented to lactate by lactic acid bacteria. The lactate was then oxidized with permanganate to acetaldehyde and carbon dioxide, and the acetaldehyde was further degraded to iodoform and formic acid As indicated in the equations, the carbon dioxide is from positions 3 and 4 of the glucose, the iodoform from 1 and 6, and the formic acid from 2 and 5

(22) $\overset{1}{CHOH}\ \overset{2}{CHOH}\ \overset{3}{C^{13}HOH}\ \overset{4}{C^{13}HOH}\ \overset{5}{CH}\ \overset{6}{CH_2OH} \rightarrow$ (ring: 1–2–3–4–5–O)

$2CH_2\ CHOH\ C^{13}OOH$

(23) $CH_2\ CHOH\ C^{13}OOH \xrightarrow{KMnO_4} CH_2\ CHO + \underset{3,4}{C^{13}O}$

(24) $CH_2\ CHO \xrightarrow{NaOI} \underset{1,6}{CHI_3} + \underset{2,5}{HCOOH}$

In the second method of degradation the glucose was converted to methyl glucoside and the latter was oxidized with periodic acid

(25) $CH(OCH_3)\ CHOH\ C^{13}HOH\ C^{13}HOH\ CH\ CH_2OH \xrightarrow[\text{Room temerature}]{HIO_4}$

$CH(OCH_3)\ CHO + C^{13}HO\ CH\ CH_2OH + \underset{3}{HC^{13}OOH}$

(26) $CH(OCH_3)\ CHO + C^{13}HO\ CH\ CH_2OH + \xrightarrow[\text{Boiling temperature}]{HIO_4}$

$\underset{1,2,4,5}{4HCOOH} + \underset{6}{HCHO} + CH_3OH$

The formic acid from the first oxidation at room temperature is from position 3 and the formaldehyde of the oxidation at boiling temperature is from position 6

The combined results from these two degradations established that the fixed carbon was in positions 3 and 4 of the glucose and that as nearly as could be judged by the methods, the concentration was the same in the two positions The latter measurement involved technical difficulties which made it subject to considerable error

Clearly these findings are in full agreement with the scheme proposed by the Harvard Group However, it is apparent that whether the fixation is essential or not, the distribution of the fixed carbon in the glycogen may be the same In other words, Reactions 19 and 20 may occur and introduce carbon dioxide in pyruvate but these reactions may have no function in the formation of phos phopyruvate All of the phosphopyruvate may actually be formed by direct phosphorylation with adenosine triphosphate as shown by Lardy and Ziegler (74)

It should be noted that the shift of the hydroxyl in enol-oxalacetate does not occur spontaneously (17) (cf Section I, p 201) and this can no longer be con sidered a mechanism for the formation of the labeled pyruvate from oxalacetate It also should be borne in mind that the carbon dioxide may be fixed by $C_2$ and $C_1$ additions (Section I) and thus give rise to carboxyl labeled pyruvate and a similar type of glycogen

There is an interesting possibility for checking whether or not the fixation of carbon dioxide in glycogen involves a symmetrical dicarboxylic acid It involves the feeding of a carbonyl or methyl labeled pyruvate If the carbon dioxide is fixed in pyruvate by Reactions 19 and 20, then from these types of pyruvate would be formed a pyruvate with labeled carbon in both the methyl and carbonyl positions and this compound would give rise to glucose labeled in positions 1, 2, 5 and 6 On the other hand, if the fixation of carbon dioxide was by direct ex-change in the carboxyl of pyruvate, and the carbonyl labeled pyruvate were used, the glucose would be labeled in positions 2 and 5 A result such as this, or one in which the concentration was greater in the 2 and 5 than the 1 and 6 positions, would be direct proof that some pyruvate was converted to glycogen without

passing through a symmetrical molecule However, even if the results showed that carbon dioxide fixation occurred by formation of a symmetrical molecule it would not prove that the reaction promoted phosphopyruvate formation

It is noteworthy that the labeled carbon was not randomized between positions 1 and 3, or 4 and 6 of the glucose in either the animal conversion to glycogen or in the bacterial degradation of the glucose Thus, at no point in glycolysis was there formed a symmetrical molecule such as dihydroxyacetone This fact is evident, for if such a compound occurred as an intermediate the orientation of the carboxyl carbon would be lost during the glycolysis and the fixed carbon would no longer be confined to only the 3 and 4 positions Thus fact proves that there was no substantial dephosphorylation of dihydroxyacetone phosphate and subsequent fermentation of the unphosphorylated compound nor was there shifting of phosphate

A problem, which in an indirect way is related to carbon dioxide fixation, is the conversion of lower fatty acids to glycogen In this conversion the question arises whether the acid is changed directly to glycogen or whether the labeled carbon is incorporated in the glycogen by carbon dioxide fixation after oxidation of the acid Buchanan *et al* (72) have investigated this problem with carboxyl labeled acetate, propionate, and butyrate fed in conjunction with glucose The radioactivity of the liver glycogen and of the expired carbon dioxide was determined, and on the basis of certain assumptions, the radioactivity of the glycogen, which was due to carbon dioxide fixation, was calculated In the case of propionate and butyrate a considerable proportion of the acids were estimated to have entered the glycogen in a form other than carbon dioxide On the other hand, with acetate the total radioactivity of the glycogen was accounted for by fixation of the radioactive carbon dioxide derived from the acetate

This problem has been investigated further by Lorber *et al* (76) The reasoning followed in their experiments was that any carbon which enters glycogen as carbon dioxide will be in the 3 and 4 positions and if labeled carbon is found in other positions of the glycogen, it establishes that the compound entered in a form other than carbon dioxide They have conducted experiments following the procedure of Buchanan *et al* (72) with the following types of acids $CH_3$ $C^{13}OOH$, $C^{13}H_3$ $C^{13}OOH$, $CH_3$ $CH_2$ $C^{13}OOH$ and $CH_3$ $CH_2$ $CH_2$ $C^{13}OOH$ The degradation procedures of Reactions 22 through 26 were used. It was found that with all three of the carboxyl labeled acids the labeled carbon was in positions 3 and 4 of the glucose of the glycogen On the contrary, with the acetate which was labeled in both positions, the $C^{13}$ was not exclusively in the 3 and 4 positions but apparently was in all the positions of the glucose This latter result is interpreted by these investigators as conclusive proof that at least *a part of the* acetate molecule enters glycogen in a form other than carbon dioxide This constitutes the first direct evidence that acetate can be converted to glycogen With regard to the findings with the three carboxyl labeled acids, the fact that the tagged carbon was only in the 3 and 4 positions was not considered evidence that this carbon entered the glycogen only in the form of carbon dioxide The calculations of Buchanan *et al* (72) indicate that at least part of the butyrate

and propionate carboxyls are not deposited as carbon dioxide This fact, together with the direct demonstration with the doubly labeled acetate (76) that acetate is converted to glycogen, makes it seem probable that propionate and butyrate may likewise be transformed to glycogen by a mechanism in addition to that of carbon dioxide fixation However, a more direct proof is to be desired

A consideration of the mechanism by which these acids are converted to glycogen will be presented in the next section, p 229, which deals with the tricarboxylic acid cycle

With regard to the calculation by Buchanan *et al* (72) of the amount of carbon dioxide fixed, it seems probable that whether or not a carboxyl labeled compound will be estimated to enter entirely by carbon dioxide fixation will depend on the rate of conversion of the compound to glycogen For example, if carboxyl labeled pyruvate was fed and the rates of Reactions 19 and 20 were rapid as compared to the rates of conversion of pyruvate to glycogen, the carboxyl of the pyruvate would come to equilibrium with the carbon dioxide and the calculations would indicate all of the pyruvate entered by carbon dioxide utilization Thus, with different carboxyl labeled compounds the proportion, which the calculation would give as entering by carbon dioxide fixation, would depend, in part at least, on the extent conditions were established so that there was little equilibration of the carboxyls with carbon dioxide

*Unidentified compounds* In the experiments by Solomon *et al* (12) only 61 per cent of the $NaHC^{11}O_3$ injected in rats was accounted for in expired $C^{11}O_2$, unabsorbed $NaHC^{11}O_3$, urine, bone, liver glycogen and body fluids The soft tissue could not be analyzed and the investigators believe that a large portion of the injected $C^{11}$ was present in it in organic combination The results of Swenseid (77) are at variance with these results She could find no evidence of such a substantial fixation of carbon dioxide in mice

Evans and Slotin (10) found in their experiments with pigeon liver acting on pyruvate that part of the fixed carbon was liberated by chloramine–T This carbon is believed to have been fixed in the carboxyl groups of amino acids

Barker *et al* (45) have obtained evidence that the methane bacteria fix carbon dioxide in protoplasm and Foster *et al* (7) have obtained similar evidence with molds

SUMMARY Carbon dioxide has thus far been shown to be fixed in methane, urea, formate, acetate, pyruvate, lactate, propionate, propyl alcohol, oxalacetate, succinate, fumarate, malate, butyrate $\alpha$-ketoglutarate, oxalosuccinate, citrate, glycogen and some as yet unidentified compounds The mechanisms of the fixation of carbon dioxide in these compounds is considered in relation to the three known primary fixation reactions

The formation of acetate and butyrate with all carbons containing fixed carbon has been demonstrated with bacteria This finding is of special interest because of its similarity to autotrophic synthesis of compounds from carbon dioxide only

The fixation of carbon dioxide in the carboxyl group of acetate by *Aerobacter* may take place via reductive cleavage of succinate Considerable evidence has

been presented indicating there is reversal of this reaction, i e , synthesis of succinate from acetate

It has been demonstrated that carbon dioxide is fixed in rat liver glycogen in the 3 and 4 positions of the glucose unit By application of this finding to acetate metabolism it has been shown that acetate is converted to rat liver glycogen in a form other than as carbon dioxide

SECTION III THE TRICARBOXYLIC ACID OR KREBS' CYCLE The tricarboxylic acid cycle is the only mechanism which has received widespread consideration as an explanation of the chemical reactions involved in biological oxidation yielding carbon dioxide and water This cycle was first proposed as a result of studies on the oxidation of pyruvate by pigeon breast muscle (78) but has gradually grown in stature until it is now receiving increasing attention as a general mechanism for the oxidation of carbohydrates, fats, and proteins by the animal body In this broad function of the cycle it is assumed that in part, at least, each of these types of substances may give rise to a common intermediate (e.g , pyruvate or an acetyl group) which is subject to oxidation in the cycle

The cycle is considered of general occurrence since in modified forms it has been suggested to apply not only to pigeon breast muscle (78), (79) but to liver (80), (81), (2), heart (82), kidney (83) (84), (85), (86) and yeast (87)

*Evaluation of the experimental basis of the scheme* The evidence and experimental proof for the tricarboxylic acid or Krebs' cycle has been reviewed by Krebs (42), Evans (1), and Werkman and Wood (2) Therefore only the principal observations which have been used in support of the scheme will be cited and the steps in the cycle where the evidence is considered incomplete will be indicated

Krebs (42) has pointed out that four observations led to the original hypothesis of the cycle in pigeon breast muscle

1 The catalytic effect of citrate on respiration
2 The synthesis of citrate from oxalacetate
3 The rapid oxidation of citrate, isocitrate, cis aconitate and $\alpha$-ketoglutarate
4 The *oxidative* formation of succinate in the presence of malonate using fumarate or oxalacetate as substrates This fact proved that there are two mechanisms for formation of succinate since formation by direct reduction via Steps[1] 2, 3, and 11 of figure 1, p 212 is inhibited by malonate

The observations listed above occur with both pigeon breast muscle (79) or liver (80) To the above points may be added the following for pigeon liver, they apparently are characteristic of liver and not of muscle because the liver can synthesize dicarboxylic acids by carbon dioxide fixation (10), (11) (81)

5 The synthesis of citrate and $\alpha$ ketoglutarate from pyruvate (80)
6 The formation of $\alpha$ ketoglutarate with fixed carbon exclusively in the carboxyl adjacent to the carbonyl group and of succinate containing little

[1] The individual conversions of figure 1 have been numbered and are referred to as Step 1 etc on the other hand the conversions which have been illustrated in the text proper are designated as Reaction 1 etc

or no fixed carbon from pyruvate and labeled carbon dioxide by pigeon liver homogenate in the presence of malonate (11), (10)

In addition there has recently been added evidence that the cycle functions in the oxidation of acetoacetate by kidney and acetate by yeast The following are pertinent points from these investigations

7 Citrate is formed from acetoacetate and oxalacetate by kidney (83), (84), (86), and from acetate and oxalacetate by yeast (87)

8 $C^{13}$-$\alpha$-ketoglutarate is formed in oxidations by kidney to which $C^{13}$-acetoacetate (85 or $C^{13}$-acetate (85) (88) is added and $C^{13}$-aspartate and glutamate are formed by the intact animal to which $C^{13}$-acetate is fed (89) The latter two compounds are important in considerations of the cycle since they are considered to be in biological equilibrium with oxalacetate and $\alpha$-ketoglutarate respectively

Whether these observations which have been obtained with different tissues and under different conditions are all the result of a similar cycle, as has been suggested, cannot be definitely answered at present It is conceivable that the scheme applies only in part to certain tissues and also that tissues have more than one mechanism for oxidation to carbon dioxide

The oxidation of pyruvate will serve to illustrate the general mechanism of the cycle, figure 1 The pyruvate is oxidized by Step 4 to carbon dioxide and an acetyl group, the acetyl group then unites with oxalacetate in Step 5 to form aconitate Aconitate is converted to isocitrate by Step 6 and it in turn is oxidized to oxalosuccinate in Step 7 The oxalosuccinate is decarboxylated yielding $\alpha$-ketoglutarate in Step 8 and this compound is in turn oxidized to succinate in Step 10 The succinate is successively oxidized to fumarate, converted to malate and then oxidized to oxalacetate in Steps 11, 3, and 2 The net result of this series of changes is the oxidation of one molecule of pyruvate to carbon dioxide and water and the regeneration of a molecule of oxalacetate which can again function in the cycle

The principal modification in the cycle, from its original form, has been to place citrate in a side reaction It became apparent that citrate was not on the direct path of the cycle, when it was found that $\alpha$-ketoglutarate, which contained fixed carbon in only one carboxyl group (11); (10), was formed by pigeon liver This observation excluded citrate as a member of the cycle, because $\alpha$-ketoglutarate as formed from citrate would contain fixed carbon equally in both carboxyls of the ketoglutarate and not in just one carboxyl Such a distribution is obligatory from citrate because of the symmetrical nature of the molecule Likewise, more recent observations have indicated that citrate is not a component of the cycle in oxidation by other tissues These results will be considered in detail in subsequent discussions of the experiments

The modified tricarboxylic acid cycle presented in figure 1 satisfactorily meets all the requirements of the eight experimental points which have been listed above and it can be used as a basis for explanation of many other observations on carbohydrate, fat and protein metabolism

The two experimental observations which have had the greatest weight in the development of the scheme were numbers 4 and 6 These points were based on

the function of malonate as an inhibitor for the reductive formation of succinate from fumarate in Step 11 Krebs has postulated the tricarboxylic cycle via Steps 5, 6, 7, 8 and 10 as the malonate insensitive mechanism of formation of succinate and considers this mechanism part of the normal oxidative process That the inhibition of malonate is effective was shown in the experiments of Wood *et al.* (11) with pigeon liver acting on pyruvate and $NaHC^{13}O_3$ When malonate was present succinate was formed which contained no excess $C^{13}$ although in the same reaction fumarate and malate occurred containing excess $C^{13}$ Thus in this case it is apparent that the succinate did not arise from the fumarate. When malonate was not added the dicarboxylic acids all contained about the same amount of $C^{13}$, indicating that in the absence of the inhibitor the dicarboxylic acids were reversibly interconvertible, the $C^{13}$-succinate arising by reduction of fumarate, Step 11 There is, therefore, clear evidence of Krebs' contention (79) that there are two mechanisms for formation of succinate, one which is not inhibited by malonate and another which is inhibited.

The origin of $\alpha$ ketoglutarate via tricarboxylic acids and the evidence of the rôle of the tricarboxylic acids in the cycle has largely been based on the following of the above listed points Point 1, the catalytic effect of citrate, Point 3, the rapid oxidation of tricarboxylic acids to $\alpha$-ketoglutarate and succinate, and Points 2 and 5, the synthesis of citrate and $\alpha$-ketoglutarate from pyruvate or oxalacetate These results definitely suggest that the tricarboxylic acids may function as intermediates but they do not establish that they are an *essential* part of the mechanism

The possibility of isocitrate not being on the main path of oxidation but being formed as a side reaction is called to attention by the results of Ochoa (19) who has shown that $\alpha$-ketoglutarate can be converted by carbon dioxide fixation to isocitrate (cf Section 1, p 206) Thus, when $\alpha$-ketoglutarate accumulates, isocitrate would also tend to accumulate and the formation of tricarboxylic acids can be accounted for Likewise the rapid oxidation of these compounds can occur by reversal of the reactions studied by Ochoa.

The key to the problem of pyruvate oxidation seems to rest on determining the mechanism of formation of $\alpha$-ketoglutarate In the case of pyruvate oxidation by pigeon liver there is an additional fact that comes into consideration, that is, that the $\alpha$-ketoglutarate contains fixed carbon only in the carboxyl adjacent to the carbonyl group

A speculative mechanism which would yield $\alpha$-ketoglutarate with fixed carbon in the proper position would be Reaction 27 involving pyruvate in which $C^{13}O_2$ has been fixed in the carboxyl group

(27) $CH_3\ COOH + CH_3\ CO\ C^{13}OOH \rightarrow COOH\ CH_2\ CH_2\ CO\ C^{13}OOH$

This reaction has been postulated for animal tissue but never proved to occur (90) It is conceivable that a reaction such as this could occur in liver in which heavy carbon pyruvate was formed via the oxalacetate $\beta$-carboxylase reaction and acetic acid or some derivative was formed by oxidation of pyruvate or fat In this scheme Step 5 would not occur and Reaction 27 would serve as a pathway to $\alpha$-ketoglutarate The oxidation of pyruvate might then be by conversion of

one molecule of pyruvate to acetate, which would in turn unite with a second molecule of pyruvate and form α-ketoglutarate The remainder of the oxidation would be Steps 10, 11, 3, 2, 1 The net results would therefore be that from two molecules of pyruvate three molecules of carbon dioxide would be formed and one molecule of pyruvate would be regenerated If such an oxidation occurred, the requirements of the above listed observations could be accounted for as follows without recourse to tricarboxylic acids as the main path of oxidation

Points 2, 3, and 5, i e , formation and rapid utilization of tricarboxylic acids, could take place by the oxalosuccinate carboxylase reaction Point 6, formation of succinate in the presence of malonate, could be readily explained, since Krebs has shown that oxidation of α-ketoglutarate to succinate is not inhibited by malonate The resulting succinate would contain no fixed carbon since the fixed carbon of the α-ketoglutarate would be split out in the oxidation Point 1, the catalytic effect of citrate on respiration might result because of the formation of $C_4$ dicarboxylic acids from the citrate by Steps 21, 6, 7, 8, 10, etc The dehydrogenation of malate to oxalacetate is one of the most rapid reactions known for tissue and thus formation of a $C_4$ dicarboxylic acid might catalyze respiration by providing a hydrogen carrier (cf Szent-Györgyi (91))

It is evident that in this scheme whenever citrate or isocitrate is formed there should be fixed carbon in these compounds With pigeon breast muscle, however, only a small amount of carbon dioxide has been observed fixed (1), and apparently Reaction 1 does not occur Fixation by oxalosuccinate carboxylase, Step 8, may occur nevertheless, for it would not be expected that there would be a large net uptake of carbon dioxide by this reaction, since the amount of citrate and isocitrate which accumulates in the oxidation is small It is to be noted that the oxalosuccinate carboxylase reaction is an essential step in the tricarboxylic acid cycle itself, so even by this cycle carbon dioxide fixation is to be expected by pigeon breast muscle

It is clear that the above comments concerning α-ketoglutarate synthesis are purely speculative The discussion is intended only as an illustration of the development and basis of the cycle and to point out that in some respects it is tentative It serves to emphasize the point of greatest experimental weakness in the Krebs cycle, i e , the initial condensation Thus far there is no *direct* proof that the tricarboxylic acids are formed by condensation of $C_3$ or $C_2$ and $C_4$ compounds and especially that this step is essential in oxidation Such proof will require a study of the isolated reaction and elimination of the possibility of synthesis of isocitrate from α-ketoglutarate by carbon dioxide fixation

The recent results of Weinhouse *et al* (88) serve to emphasize the uncertainty regarding the initial condensation These authors find, when carboxyl labeled acetate is oxidized by kidney slice in the presence of citrate, cis-aconitate, or α-ketoglutarate, that isotopic carbon is incorporated in the α-ketoglutarate but not in the citrate This latter result was obtained when the citrate was added as such, or when aconitate was added and the citrate formed from aconitate was isolated Weinhouse *et al* (88) have concluded that citrate, isocitrate, and

aconitate are not in the direct path of acetate oxidation by kidney They suggest the possibilities (a) that the tricarboxylic acid may be a phosphorylated derivative not in equilibrium with the parent substance, (b) that dehydrogenating coupling between oxalacetate and acetate might yield oxalosuccinate directly, (c) that acetate may couple with pyruvate directly to yield $\alpha$ ketoglutarate without intervention of tricarboxylic acids

It is clear that these results are a serious challenge to the occurrence of tricarboxylic acids as intermediates in the cycle In the author's opinion the results would be more convincing if aconitate or isocitrate as such were isolated It has been generally accepted that citrate is not an intermediate in the tricarboxylic acid cycle (10), (11), therefore it is questionable if this substance should be used as representative of the tricarboxylic acids It is conceivable that the cis aconitate, when added in high concentration, is rapidly converted to citrate and that subsequently the interconversions are not favorable for incorporation of $C^{13}$-acetate into the citrate

The tricarboxylic acid cycle is the most complete explanation that has been given of biological oxidation up to the present date, and so far, with only minor modification, it has proved adaptable to all new developments The scheme, therefore, should form a basis for future studies and can be modified to fit developments as they arise

The remaining portion of this Section will be limited to a discussion of the cycle in relation to its application to specific problems and investigations

*The tricarboxylic acid cycle and oxidation of carbohydrate.* In the scheme presented in figure 1 an attempt is made to give an integrated picture of the interrelationships of carbohydrate, fat and protein metabolism The scheme, of course, is highly speculative and leaves many questions untouched Nevertheless, a purpose is served in that it emphasizes an idea that is becoming increasingly apparent, that is, that fat, protein and carbohydrate metabolism have many points in common in the form of identical intermediate products (cf Krebs (42)) Through these intermediates, interconversion of fats, carbohydrates and proteins may occur, and likewise through them oxidation may occur, in part at least, by essentially the same mechanisms The "switch" from carbohydrate to fat oxidation, which is frequently spoken of, probably does not represent a major shift in mechanism but rather a change which is manifested through the same mechanism and may be produced by the differences in the degree of oxidation of the different substrates—carbohydrates as compared to fats For example, carbohydrates and fats may both give rise to acetyl groups via Steps 4 and 18 and the acetyl group arising from either substrate may be oxidized by an identical mechanism The fact that acetone bodies are formed from fat does not necessarily imply that there has been a switch in the fundamental mechanism of oxidation but rather it may imply that acetone bodies are formed from fat faster than they are broken down. This may occur because the rate of formation of acetyl groups from fat may be more rapid than is the oxidation via the cycle and the acetyl groups are therefore converted to acetone bodies Likewise a respiratory quotient of 1 0

probably does not indicate that fat is not turning over and is not contributing to the oxidation product $CO_2$ but rather, it indicates that the net metabolism is equivalent to carbohydrate oxidation

In considering the experimental basis for the cycle, some consideration already has been given to carbohydrate, i e , pyruvate oxidation For the present purpose it will be sufficient to consider the scheme only in relation to the isotope studies with labeled carbon dioxide These investigations have, so far, been done almost entirely with pigeon liver, fixation by other animal organs, except heart (13), (19) has not been demonstrated With pigeon liver acting on pyruvate, carbon dioxide is fixed in $\alpha$-ketoglutarate (10), (11) and in the carboxyls of malate, fumarate, succinate and lactate (11)

In the cycle (fig 1) starting with the conversion of pyruvate to oxalacetate by oxalacetate carboxylase (Step 1) the labeled carbon dioxide is fixed in the carboxyl adjacent to the methylene group of oxalacetate By the reversible conversion of oxalacetate to fumarate, Steps 2 and 3, fixed carbon dioxide is distributed in both carboxyls of these $C_4$ dicarboxylic acids If malonate is not present to inhibit the conversion of fumarate to succinate, the fixed carbon is likewise distributed in the succinate The carboxyls marked "b" represent carboxyls which contain fixed carbon and if the rates of the reactions are adequate these carboxyls are in equilibrium with the carbon dioxide The reversible conversion of oxalacetate to pyruvate and its reduction to lactate (Step 16) accounts for fixation in this compound Likewise by the reversible reactions, the $\alpha$ and $\beta$ carbons of the pyruvate lose their orientation in oxalacetate, and the carbons marked "a" theoretically represent the average of these two carbons This latter point has not been checked as yet with an isotope tracer of these carbons

Because there is some evidence in fatty acid oxidation that a $C_2$ compound enters the cycle (85), (88), (89) the primary condensation reaction, Step 5, is pictured as $C_2$ and $C_4$ addition to aconitate The $C_2$ compound, which may be acetyl phosphate, is considered formed from pyruvate and fat by oxidation

Krebs (92) has presented evidence that little exchange is to be expected between citrate and aconitate with pigeon liver because the rate of equilibration between citrate and cis-aconitate (Step 21) is slow as compared to the rapid rate of conversion of cis-aconitate to $\alpha$-ketoglutarate This observation is believed to account for the finding of substantially all the fixed carbon in only one carboxyl of the $\alpha$-ketoglutarate (10), (11) If Step 21 was rapid all three carboxyls of the tricarboxylic acid would contain fixed carbon and the $\alpha$-ketoglutarate formed by Step 8 would have fixed carbon in both carboxyls

It is interesting that the discovery of fixation of carbon dioxide by the oxalosuccinate carboxylase reaction (Step 8) has not necessitated revision of any ideas on the cycle or invalidated any previous conclusions It can be seen that Step 8 to the left inserts carbon dioxide into a carboxyl position, which already contains fixed carbon by virtue of the primary fixation (Step 1) and equilibria, Steps 2 and 3, and the additional, Steps 5, 6, and 7 The fixation by Step 8, thus, does not change the distribution of fixed carbon, except insofar as the rates may have been inadequate to equilibrate the carboxyls with carbon dioxide

The oxidation of α ketoglutarate to succinate, Step 10, eliminates the second "b" carboxyl and therefore the succinate formed from this compound contains no fixed carbon

It is to be noted that the regenerated oxalacetate does not have fixed carbon introduced into its methylene and carboxyl groups Therefore, repetition of the cycle cannot cause a new distribution of the fixed carbon in the components of the cycle. The cycle, thus, accounts for the known facts about the location of fixed carbon in components of the cycle

No mention has been made in this discussion of the cycle about the occurrence of phosphorylated compounds There is no definite information available on this subject although there is evidence that phosphorylation may occur A phosphorylated compound may be formed in the oxalacetate β-carboxylase reaction, since ATP is a component of the system (25) The conversion of pyruvate, Step 4, may involve the formation of acetyl phosphate (33) but this reaction has not been verified with animal tissue Furthermore, Ochoa (93) has found that in the oxidation of pyruvate to carbon dioxide by heart muscle extract, three atoms of phosphate are esterified per atom of oxygen utilized The esterified phosphate was detected as ATP or hexosemonophosphate This esterification is equivalent to fifteen atoms of phosphate per molecule of pyruvate The same ratio of phosphate esterified to oxygen utilized has been observed by Ochoa (94) in the oxidation of the α-ketoglutarate to succinate In the oxidation of succinate to fumarate the ratio is less and not more than one molecule of phosphate is esterified per atom of oxygen (95) The oxidation of malate to oxalacetate likewise causes phosphate uptake (96)

While there is little question that phosphate may be esterified in the oxidations of the cycle, there is little known about the mechanisms Although part of the phosphate may enter into esterification with the components of the cycle during their dehydrogenation, it is not likely that more than a part of the phosphate is used for this direct phosphorylation of the substrate No mechanism is known for such a large utilization of phosphate by this means Part of the phosphate is probably used during the intermediate steps of transfer of the hydrogen to oxygen (93)

The mechanism of hydrogen transfer in the cycle has been studied by Krebs (97), (42) He has concluded that possibly all but one pair of the hydrogens pass through malate by the hydrogen carrier cycle of Szent-Györgyi (91) However, Ochoa (94) has now studied the one step concerning which Krebs was in doubt, i.e , the oxidation of α-ketoglutarate to succinate and has shown that the $C_4$ dicarboxylic acids are not involved in this oxidation Therefore, it seems probable that only four pairs of hydrogen can be transferred by the malate-oxalacetate system in the tricarboxylic acid cycle

In view of Ochoa's discovery of a new fixation reaction in the cycle it is interesting to consider whether fixation may occur in any of the other reactions It will be noted that two Steps, 1 and 8, of the four decarboxylations have been shown to be reversible with animal tissue On the other hand, Steps 4 and 10, the oxidation of pyruvate to a $C_2$ compound and of α-ketoglutarate to succinate have not

There is some indirect though not conclusive evidence that these reactions are not reversible In the experiments of Wood *et al* (11) with pigeon liver, succinate was formed which contained excess $C^{13}$ in the carboxyl groups If Step 10 were reversible and α-ketoglutarate were formed from succinate, the excess $C^{13}$ of the succinate would be incorporated in the α-ketoglutarate This conversion apparently did not occur because there was excess $C^{13}$ present in only the one carboxyl group, none being present in those carbons that would be derived from the succinate Apparently if the reaction is reversible the rate was too slow to be detected under the conditions of this test

The determination of the irreversibility of Step 4 is not direct and is not nearly as conclusive In the experiments of Lorber *et al* (76) in which $CH_3C^{13}OOH$ was fed to rats the excess $C^{13}$ was found exclusively in the 3 and 4 positions of the glucose unit of the liver glycogen However, if Step 4 were reversible, it would be expected that pyruvate would result with excess $C^{13}$ in the carbonyl group If this compound were converted to glycogen, this excess $C^{13}$ would be incorporated in the 1, 6 and 2, 5 positions of the glucose (cf Section II, p 218) Actually the dilution of the labeled carbon is so great in these experiments that the sensitivity of the test is low There was a consistent slight excess of $C^{13}$ in the 1, 6, 2, 5 positions of the glucose from these experiments, but it was less than the experimental error of the methods and was therefore not held to be significant If Step 4 is reversible, it seems from these results that the rate must be quite slow as compared to the incorporation of the labeled carbon into the 3 and 4 positions The concentration of $C^{13}$ in these positions was definitely significant

*The tricarboxylic acid cycle and glycogen synthesis* The problem of glycogen synthesis and fixation of carbon dioxide has been considered in Section II, p 216 and at this point the discussion can be limited to a consideration of the application of the findings to the tricarboxylic acid cycle

With regard to the fixation of carbon dioxide in the 3 and 4 positions of glucose, it is evident that if Steps 1, 2 and 3 are reversible then pyruvate containing fixed carbon will be formed It seems probable that part of this pyruvate will be converted directly to glycogen by reversal of glycolysis through Steps 15, 13, and 14 However, part of the pyruvate probably enters the glycogen after going through the tricarboxylic acid cycle Since the dicarboxylic acids which are formed from α-ketoglutarate contain no fixed carbon, this mechanism would not provide any fixation of carbon dioxide in the glycogen

Whether the $C_4$ dicarboxylic acids are in part converted to phosphopyruvate by some mechanism other than conversion to pyruvate and phosphorylation of the pyruvate with ATP (74) is not certain Because Step 15 was believed to be irreversible (73), considerable speculation was aroused concerning alternate pathways for the formation of phosphopyruvate The reaction which received the most attention for this transformation was the oxidation of fumarate Kalckar (98) had demonstrated phosphopyruvate synthesis in fumarate oxidation and Lipmann (33) proposed a mechanism for this synthesis involving addition of inorganic phosphate to fumarate followed by oxidation to phospho-enoloxal acetate and then decarboxylation to phosphopyruvate Carbon dioxide fixation

posed to play an essential rôle in this mechanism via Steps 1, 2, 3 and 12
tion II) However, Lardy and Ziegler's (74) findings of the reversibility
15 have now removed the essential requirement for the fixation reaction
ver, the fact that Lelοir and Munoz (99) have found that adenylic acid is
ry for formation of phosphopyruvate from succinate fumarate, or citrate,
es that a phosphate transfer, rather than a direct uptake of inorganic
ate may occur in this formation of phosphopyruvate In this case, the
on of the $C_4$ dicarboxylic acids may simply function to synthesize ATP for
osphorylation of pyruvate or of $C_4$ acids At present the occurrence
phopyruvate formation by addition of inorganic phosphate *via* Step 12
e considered speculative It seems probable that carbon dioxide is fixed
ogen by reversible equilibria 1, 2, 3, 15, 13, and 14, and that the obligatory
n of carbon dioxide in glycogen synthesis is to be questioned
ough the oxidative pathway of the tricarboxylic acid cycle does not
direct part in the incorporation of fixed carbon into glycogen except as it
pply the energy for this synthesis, it seems likely to have a more direct
n in the conversion of acetate to glycogen Lorber *et al* (76) (cf Section
19) have shown that carboxyl labeled acetate fed to rats yields liver glyco-
th labeled carbon in the 3 and 4 positions of the glucose unit With ace
ntaining tagged carbon in both carbons of the acetate, the isotope was
ntly in all positions of the glycogen (76)
e acetate carbon is traced through the cycle of figure 1 it is clear that the
ed distribution of labeled carbon results. The Steps are 19, 5, 6, 7 8,
3, 2, 1, 15, 13, and 14 At the α-ketoglutarate step the carboxyl labeled
would give an α ketoglutarate with tagged carbon in the carboxyl adja
the methylene group This compound upon oxidation yields succinate
e labeled carbon in the carboxyl groups Then in Step 1 carboxyl labeled
te would be formed and this compound would yield glycogen with tagged
in the 3 and 4 positions of the glucose
the other hand, with the acetate with both carbons labeled, α-ketoglu
would result with the carboxyl group and adjacent methylene group
l Succinate then would be formed, Step 10, with labeled carbon in the
yl and methylene groups At Step 1, pyruvate would arise with tagged
in all positions, and from this pyruvate glucose would be formed with all
ns labeled The $C^{13}$ was not found to be uniformly distributed in the
e, however In one experiment (76) for example, the following values
btained for the excess $C^{13}$ of the glucose 3 and 4 positions 0.36, 2 and 5
ns 0.28 and 1 and 6 positions 0.22
lanation of this variation of the $C^{13}$ is not difficult insofar as the 3 and 4
ns are concerned since the acetate carbon probably enters these positions
o mechanisms, i.e , by fixation of the $C^{13}O_2$ arising from the oxidation
acetate and also by incorporation of the acetate carbon directly via the
boxylic acid cycle as outlined above The combined effect of these two
nisms could be to raise the $C^{13}$ in the 3 and 4 positions above that of the
carbons of the glucose

The explanation of the cause of the difference between the $C^{13}$ content of the 2 and 5 positions and 1 and 6 positions is not nearly as evident The $\alpha$ and $\beta$ positions of the pyruvate formed by Step 1 of the scheme arise from the methylene carbons of the succinate Since the succinate molecule is symmetrical, the $C^{13}$ from the methylene carbons would of necessity be uniformly distributed in the $\alpha$ and $\beta$ positions of the pyruvate and furthermore, via Steps 15 and 13, the distribution would be uniform in positions 1 and 6 and 2 and 5 of the glucose Lorber, *et al* (76) have therefore concluded that the tricarboxylic acid cycle as given in figure 1 may not be the sole route of conversion of acetate to glycogen They point out, however, that the results can be accounted for by the tricarboxylic acid cycle if the scheme is modified One modification that is suggested is of Steps 11, 3 and 2 of figure 1 to provide a pathway involving unsymmetrical molecules This pathway may be in the form of phosphate esters of succinate and fumarate However, it would seem necessary to assume that these unsymmetrical forms are in partial equilibrium with the symmetrical acids This suggestion is indicated since the $C^{13}$ concentration is not greatly different in the 1 and 6 and 2 and 5 positions, and therefore some randomization mechanism must be operating Likewise in the scheme for carbon dioxide fixation in glycogen it has, thus far, been found necessary to assume that the oxalacetate is in at least partial equilibrium with a symmetrical molecule This mechanism gives the necessary randomization of the fixed carbon in the carboxyl groups of oxalacetate so that pyruvate as formed from oxalacetate will contain fixed carbon

It is obvious that the above explanation of acetate conversion to glycogen is entirely tentative Of course, other schemes can be devised to fit the results For example, $\alpha$-ketoglutarate might be formed from pyruvate and acetate by Reaction 27 This $\alpha$-ketoglutarate would be similar to that formed by the tricarboxylic acid cycle and thus would have the same limitations For the present, however, since the tricarboxylic acid cycle is far more complete than other mechanisms, it seems advisable to use this cycle as a basis for speculation

The conversion of carboxyl labeled butyrate and propionate to liver glycogen likewise, was studied by Lorber *et al* (76) and the labeled carbon was shown to occur in the 3 and 4 positions of the glucose unit The mechanism of these transformations is not definitely known, but in the case of butyrate, conversion to acetoacetic acid and then to an acetyl group seems the most likely mechanism (cf Fatty acid oxidation, p 236) According to this proposal the acetyl group would enter the tricarboxylic acid cycle by the same reactions as carboxyl labeled acetate, and give rise to glucose with the tagged carbon in the 3 and 4 positions

Blixenkrone-Möller (100) has suggested that the butyrate is converted to succinate by omega oxidation and that this is the reason butyrate is glycogenic The change of succinate to glycogen most likely would be by oxidation to pyruvate through Steps 11, 3, 2, and 1, and this transformation, just as in the tricarboxylic acid cycle (via Step 15, etc ), would place the carboxyl carbon of the butyrate in the 3 and 4 positions of the glucose However, preliminary experiments by Wood *et al* (101) have indicated that this mechanism is not in agreement with their experimental observations When they fed $CH_3\ C^{13}H_2\ CH_2$

$C^{11}OOH$ to rats, the tagged carbon was found predominantly in the 3 and 4 positions whereas by the omega oxidation with succinate as an intermediate, the tagged carbon would be in all positions of the glucose On the other hand, the results are in complete agreement with the predictions for conversion to two acetyl groups and transformation via the tricarboxylic acid cycle The experiments are being repeated with butyrate containing a greater concentration of $C^{11}$ than was available in the preliminary experiments The sensitivity of the test will thus be greatly increased

The mechanism for conversion of propionate to glycogen is generally considered to be via oxidation to pyruvate The results of Lorber *et al* (76) are in agreement with this concept

Some discussion is perhaps desirable concerning the results obtained in isotope experiments in relation to past measurements of the glycogenic properties of compounds The method used in the past has been to determine the *net* increase in glycogen after feeding a compound However, it is now certain that the net increase in glycogen is not necessarily a measure of the amount of the fed compound which is incorporated in the glycogen For example, Vennesland *et al.* (71) found when $C^{11}H_3$ $C^{11}HOH$ COOH was fed to rats that the liver glycogen deposited averaged 21 per cent of the fed lactate, but contained only 3.2 per cent of the lactate radioactivity This fact emphasizes that when a compound is fed and an increase is found in a body component, it does not follow that the carbon of the fed compound is transferred as such to the increased component

Schoenheimer and Rittenberg (102) have presented evidence that the components of the body are in dynamic equilibrium and are constantly "turning over" Therefore, when a compound is fed it enters this metabolic "pool" and is converted into many different components of the body If the fed compound enters into the dynamic equilibria of those compounds which are precursors of glycogen, it is to be expected that the compound will become mixed with the other precursors in the metabolic pool Vennesland *et al.* (71) believe this dilution by mixing with precursors is the explanation for the small incorporation of $C^{11}$ from the $C^{11}$-lactate, as compared to the much greater observed net increase in the liver glycogen

Actually, the feeding of a compound could influence a body component in any one of the following ways

a. The compound fed could be transformed into the body component and a *net* increase in the component occur

b The compound fed could be transformed into the body component but *no net* increase in the component occur

c The compound fed could cause a net increase or decrease in the body component without itself entering the body component

In the latter case the fed compound would not enter the metabolic pool of precursors which serve as a source of carbon for formation of the body component, but rather by indirect action would influence the steady state of the dynamic equilibria involved in formation of the body component Presumably such a case could arise when the fed compound produced high energy phos

phate such as ATP which was used in turn in synthesis of the body component. Viewed in the light of these considerations the explanation of Buchanan *et al* (85), of why fed acetate does not usually lead to a net increase in glycogen, may be an over simplification They propose that acetate is oxidized in the tri carboxylic acid cycle to two molecules of carbon dioxide and that one molecule of oxalacetate is regenerated Since there is no net increase in the oxalacetate, which is considered to be the carbohydrate precursor, no net increase in glycogen is considered likely to occur This view overlooks the fact that the dynamic equilibrium may be subject to change not only because of the concentration of the actual carbon precursors, but also, for example, because of the energetics of the system, ATP concentration, etc

It is clear from the above discussion that the amount of isotope incorporated into glycogen likewise is not a direct measure of the net increase in glycogen. This erroneous assumption has been made by Deuel *et al* (103) in interpreting the experiments of Buchanan *et al* (72) on butyrate conversion to glycogen. Furthermore, the finding of Lorber *et al* (76) that $C^{13}$-acetate is converted to glycogen does not conflict with the results of feeding experiments which in general, have been considered to indicate that acetate is non-glycogenic (104), (105) It is only necessary to assume that when acetate is fed and enters glycogen it does not shift the dynamic equilibria in such a way as to cause a *net* increase in glycogen

*The tricarboxylic acid cycle and oxidation of fatty acids* The oxidation of fatty acids via the tricarboxylic acid cycle has only recently been given serious consideration This particular phase of fat oxidation will be considered in some detail in this review but for a more complete account of the general subject of fatty acid metabolism see Stadie's recent review (106)

The evidence of fatty acid oxidation by the tricarboxylic acid cycle has been the result of accomplishments in a number of different fields, and much additional work is necessary before uniform agreement can be reached on the mechanism of the reactions The greatest divergence of opinion concerns the type of compound which is formed in the original condensation to give a tricarboxylic acid

Consideration of the subject of fatty acid oxidation by the tricarboxylic acid cycle will take the following order

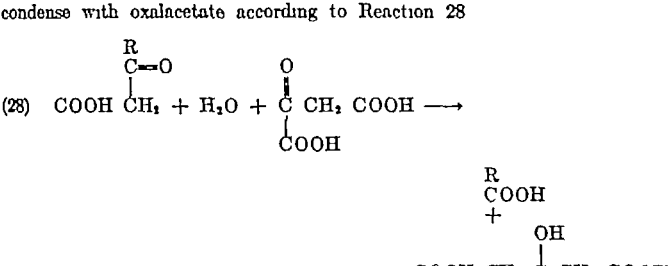
a The initial condensation reaction will be discussed, particularly the evidence will be considered in relation to a possible condensation of $C_2$ and $C_4$ compounds

b Experiments will be cited which show that $C_2$ compounds are rapidly formed from fatty acids, therefore the oxidation of fatty acid via the tricarboxylic acid cycle would not be limited by this reaction

c The evidence will be presented which shows that acetate and acetoacetate enter into formation of the intermediary compounds of the cycle, thus providing evidence that the $C_2$ component as formed from fat may be oxidized by the cycle.

a *The initial condensation reaction* The three mechanisms for the primary condensation proposed by Breusch (83), Lynen (87), and Wieland and Rosenthal (84) have received the most attention

Breusch (83) suggested that the fatty acids, after conversion to β-keto acids, condense with oxalacetate according to Reaction 28



Breusch called the enzyme citrogenase Citrogenase is presumed specific for β-keto acids but not for the R group For example, several straight and branched chained monocarboxylic, as well as dicarboxylic β-keto acids are stated to yield citrate with the enzyme Furthermore, the R COOH acid resulting from Reaction 28 after undergoing a second β-oxidation is suitable for the additional oxidation by Reaction 28 Thus complete oxidation of the fatty acid can be accounted for by the scheme

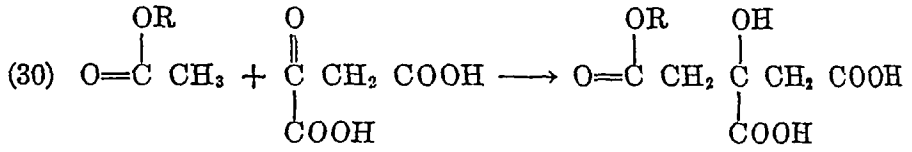
The enzyme, citrogenase, was extracted with 0 5 per cent $NaHCO_3$ and was found in large amounts in muscle, kidney and brain, but there was little in liver and none in spleen, pancreas and lung Unfortunately only the single report which contained no experimental data has thus far been available for evaluation of this work.

Breusch apparently does not believe that the tricarboxylic acid cycle is important in carbohydrate oxidation The basis of his criticism is not clear to the author It is very difficult to understand how he can justify the scheme for fat oxidation and in turn criticize it as an explanation for carbohydrate oxidation The fact that evidence has been obtained that citric acid is not a component of the mechanism of pyruvate oxidation is not a logical reason to discard the entire scheme for carbohydrates

The second mechanism of condensation was proposed by Lynen. Lynen studied the oxidation of acetate by yeast and has presented evidence that the oxidation occurs via the tricarboxylic acid cycle (107), (87) The inhibition of yeast oxidation by malonate (87) was demonstrated for the first time This was accomplished by lowering the pH to about 2, at this pH the yeast cell is permeable to malonate

Lynen suggested that the oxalacetate and acetate condensation is coupled with the dehydrogenation of aldehydes (107) An R group (perhaps a phosphate) is suggested to be linked either to the carboxyl of acetate or of oxalacetate This combination with R is believed to activate the acid so that condensation

can occur On the assumption for purposes of illustration that the acetate is the molecule that is activated, then the reaction would be as follows



(30)

It is suggested that the procitrate, thus formed, is converted to a proaconitate and then by splitting off the R, aconitate is formed The remainder of the scheme is by the usual conversions of the tricarboxylic acid cycle

The advantage claimed for this scheme is that it provides an unsymmetrical citrate molecule which makes possible the explanation of earlier results obtained by Sonderhoff and Thomas (108), with $CD_3$ COOH The results of Sonderhoff and Thomas have been discussed previously on p 212 Likewise the scheme can be used to explain the occurrence of fixed carbon dioxide in only one carboxyl of α-ketoglutarate as found in the pigeon liver experiments (10), (11) This is because the scheme avoids the randomization of the fixed carbon which would occur if the symmetrical citrate was an intermediate

Wieland and Rosenthal (84) have proposed a third mechanism for the condensation reaction leading to formation of tricarboxylic acids Wieland and Gottfried (109) had found that acetate is rapidly oxidized by kidney cortex, and since it was known that acetate could be converted by some tissues to acetoacetate, the oxidation of this latter compound was studied next (84) With kidney slices and a combined substrate of oxalacetate and acetoacetate, oxygen uptake was increased 40 per cent above the endogenous rate, whereas either substrate by itself caused only a 10 per cent increase When barium ions were added to inhibit citrate breakdown, considerable citrate was found formed

On the above basis Wieland and Rosenthal postulate that oxalacetate and acetoacetate can combine to form either citroylacetate or acetyl citrate These compounds on hydrolysis would yield acetate and citrate Since acetate was not found as an end product it was suggested that the product of the first condensation with oxalacetate might combine with a second molecule of oxalacetate to give a double condensation compound which on hydrolysis would yield two molecules of citrate and no acetate

The yield of citrate with oxalacetate and pyruvate was about 50 per cent as large as with acetoacetate It was suggested that pyruvate might first be converted to acetoacetate and then form citrate

By conversion of the unsymmetrical addition products to aconitate, the fixation experiments and deuteroacetate results of Sonderhoff and Thomas could be explained just as in the case of Lynen's scheme

The enzyme catalyzing this reaction was found in kidney and heart but not in liver

It is obvious on critical examination of the above results that the proposed mechanisms for citrate formation are purely speculative Actually, all that is known is that citric acid is formed from oxalacetate when in combination with a

number of other compounds such as *acetate, pyruvate, acetoacetate and other β-keto acids*

In practically every case oxygen was found to facilitate the reaction, whereas no function has been indicated for any oxidation in the reaction.

The fact that liver does not form citrate from acetoacetate (83), (84) has been considered an indication that liver lacks citrogenase, whereas it is just as probable that the liver preparations only lack enzymes for converting acetoacetate to an intermediate capable of taking part in the citrogenase reaction At any rate, there is an enzyme in liver which forms citrate from pyruvate and oxalacetate and there is no reason to believe it is different than the enzyme which produces citrate in kidney and heart In fact, until evidence is presented to the contrary, it is probably a fair assumption that the same enzyme causes condensation in each case

The results of Hunter and Lelour (86) are of considerable interest in this connection They have recently demonstrated with a washed suspension of kidney cortex that α-ketoglutarate oxidation stimulates the conversion of acetoacetate and oxalacetate to citrate This function of α-ketoglutarate is reminiscent of Lynen's (107) observation of the activating effect of oxidation of succinaldehyde on citrate formation by yeast On the other hand, Hunter and Lelour found that α-ketoglutarate oxidation was not necessary for the formation of citrate when oxalacetate was the only substrate Oxalacetate breaks down to pyruvate spontaneously and the reaction in the latter case may have been by union of oxalacetate and pyruvate or a product formed from pyruvate The important point is that acetoacetate apparently undergoes some primary reaction facilitated by α-ketoglutarate oxidation before it can be converted to citrate It therefore seems quite unlikely that acetoacetate is a *direct* intermediate in the citrogenase reaction

Additional evidence that acetoacetate is not an intermediate has likewise been presented by Lehninger (110) Using a liver preparation he found that citrate was formed oxidatively from either pyruvate or octanoate, when added in combination with fumarate On the other hand, when fumarate was omitted, citrate was not formed, but there was increased formation of acetoacetate as compared to that formed when the fumarate was added. From acetoacetate and fumarate the formation of citrate was no greater than from fumarate alone These results are interpreted as evidence that acetoacetate is not a component of the citrate reaction but is formed when oxalacetate is not present to shift the reaction toward formation of citrate It is suggested that pyruvate and fatty acids may have a common $C_2$ intermediate which react in the condensation

In connection with pyruvate conversion to citrate, it is to be noted that Martius (111) has presented evidence that the 7 carbon compound, 2-keto-4-hydroxyadipic acid is not precursor of citrate This compound was not converted to citrate by kidney preparation. It is of interest that Bloch and Rittenberg (112) have presented evidence that pyruvate as such may react with amines in acetylation and that the pyruvate is broken down to an acetyl group after combination with the amine It is conceivable that the same type of reaction

may occur in the condensation of pyruvate to form tricarboxylic acids, i e , the pyruvate may react prior to formation of the $C_2$ fraction

Hunter and Lelour (86) and Lehninger (110) have tested acetyl phosphate as a probable $C_2$ component for the citrogenase reaction and have found it inactive However, since acetyl phosphate is rapidly broken down by animal tissue (41), it is questionable whether the test will have any definite significance, until it is demonstrated that the ester was sufficiently stable under the conditions of the test and available for the enzyme reaction

It is evident that information concerning the initial condensation reaction is in a very confused state at present It is obvious that considered as a group, the compounds (acetate, pyruvate, acetoacetate) which form citrate in combination with oxalacetate have one thing in common, i e , they are all potential sources of $C_2$ compounds Further investigation is needed, however, before any exact idea can be reached as to the mechanism of the reaction

b *Evidence of formation of $C_2$ compounds in the oxidation of fatty acids* An interesting feature of biochemistry, that has become apparent from recent isotope studies, is the remarkable ability of animals to construct complex compounds from small units, such as $C_2$ and $C_1$ compounds There is increasing evidence that the food is dissimilated and then synthesized to body constituents Of course there are many exceptions, the essential vitamins and amino acids must be added as more complicated molecules Even here it is often true that specific groups are essential components rather than the whole molecule It is very significant that acetic acid is incorporated into such compounds as cholesterol (113), (112), hemin (112), fatty acids (114), glycogen (76), glutamate and aspartate (89) and that glycine is converted to glycogen (115), porphyrin (116) and creatine (117) Furthermore, Bloch and Rittenberg (112) have recently calculated that from 0 9 to 1 2 grams of acetate may be formed daily per 100 grams of rat tissue (cf p 239 for further discussion) Thus the $C_2$ compound clearly plays an important part in animal metabolism

The evidence that $C_2$ compounds are formed from fatty acids dates back to Knoop's studies of $\beta$-oxidation (118) Schoenheimer and his collaborators have extended these observations with the more nearly natural compounds, the deuterio labeled acids, and have demonstrated that the long chain acids are built up and broken down by units of 2 carbons For example, deuterio-palmitic acid, 16 carbons, was converted to deuterio-stearic acid, 18 carbons, and deuterio-stearic acid was converted to deuterio-palmitic acid (119, 120)

Very recently Rittenberg and Bloch (114) have presented convincing evidence that acetate is converted to fatty acids Sodium acetate containing 77 per cent deuterium in the methyl group and 19 6 per cent $C^{13}$ in the carboxyl group was fed to mice in a high carbohydrate, low fat diet at the rate of 1 6 mM of acetate per 100 g of weight per day for eight days The respiratory carbon dioxide at the conclusion of the experiment contained 0 066 per cent excess $C^{13}$ and the deuterium concentration in the body fluids was 0 09 per cent excess The fatty acids were isolated from the liver and carcass The total fatty acids of the carcass contained 0 081 per cent $C^{13}$ and 0 13 per cent deuterium while the fatty

acids of the liver contained 0 103 per cent $C^{13}$ and 0.32 per cent D These results show that both the deuterium and $C^{13}$ labeled carbon atoms of the acetate were used in the synthesis of fatty acids Since the respiratory carbon dioxide contained less $C^{13}$ than the fatty acids it was concluded that not all of the carbon of the acetate was incorporated as carbon dioxide [4]

The saturated fatty acids were isolated and then decarboxylated The concentration of $C^{13}$ in the carboxyl carbon of the saturated acids was found to be almost twice as high as the average $C^{13}$ of the entire molecule The most plausible distribution which would explain these results is one in which the isotopic carbon is in every other carbon atom, i e , the odd carbon atoms of the fatty acid This proposal is supported further by the fact that when the unsaturated "oleic acid" fraction was oxidatively split into "pelargonic" and azelaic acid fractions and analyzed, both the $C^{13}$ concentrations and deuterium concentrations were nearly the same in the two fractions Thus the isotopes were distributed in the whole carbon chain This fact indicates the complete molecule was formed with incorporation of acetate carbon Rittenberg and Bloch have estimated that at least one fourth of all the carbon atoms of the synthesized fatty acids were derived from acetate carbon

These findings can be taken to indicate that the reactions of fatty acid degradation to $C_2$ compounds are reversible, and that fats may be synthesized by these reversible reactions The condensation of acetate to acetoacetate may be the first step in the synthesis The fact that Swendseid *et al.* (121) have demonstrated *in vivo* that acetate is converted to acetone bodies gives support to this idea

It is interesting that Wood *et al* (122) obtained convincing evidence with $CH_3C^{13}OOH$ that the butyl alcohol bacteria convert acetate to butyric acid and butyl alcohol The reaction is similar to the above condensation since the $C^{13}$ was equally distributed in the carboxyl and beta positions Also the results of Barker, Kamen and Bornstein (137) are significant in this connection Using *Cl kluyveri*, which ferments alcohol and acetate to butyrate and then to caproate,

(31) $$CH_3\ CH_2OH + CH_3\ COOH \rightarrow CH_3\ CH_2\ CH_2\ COOH + H_2O$$

(32) $$CH_3\ CH_2\ CH_2\ COOH + CH_3\ CH_2OH \rightarrow CH_3\ (CH_2)_4\ COOH + H_2O$$

[4] Although the above conclusion is very likely correct it should be pointed out that based on this single observation there would be a possibility of error It is conceivable, that the rate of exchange at the site of oxidation of acetate and synthesis of fat may not be rapid enough to bring the components involved into complete equilibrium with those of the body in general In other words, the concentration of $C^{13}$ and D in the bicarbonate and water respectively of the organ or site where the oxidation is occurring might be higher than that of the respiratory carbon dioxide and average body water In this case an isotopic compound could be synthesized by incorporation of $C^{13}O_2$ and HDO and have a higher content of $C^{13}$ and D than that of the respiratory carbon dioxide and body water The possibilities of such an error are emphasized by Kalckar *et al* (140) and Furchgott and Shorr (141), who showed that the isotopic concentration in the intracellular and extracellular phosphate could be quite different and that this difference probably accounts for the observations by Sacks (142) which seemed to indicate that the Meyerhoff Warburg scheme of glycolysis did not apply to the working muscle

it was shown that in the presence of acetate labeled with $C^{14}$ in the carboxyl group, the $C^{14}$ was approximately equally distributed between the carboxyl and beta carbons of the butyrate In the caproate one-third of the $C^{14}$ was in the carboxyl group, the rest was presumably in the beta and delta positions As the fermentation proceeded the concentration of $C^{14}$ in the acetate dropped, which indicated there was oxidation of the ethanol to acetate or a compound in equilibrium with the acetate When the organism was grown in normal ethanol and carboxyl labeled butyrate, isotopic caproate was formed but not acetate The $C^{14}$ in the caproate was not in the carboxyl but presumably was in the beta position The condensation therefore involves butyrate or a related $C_4$ compound and the methyl group of acetate or a reactive derivative formed by oxidation of alcohol This bacterium thus produces a reaction which is very similar to the conversion which occurs in fatty acid synthesis by animals

Closely related to the above considerations is the mechanism of acetoacetate formation from fatty acids The original $\beta$-oxidation theory assumed that there was successive splitting off of $C_2$ units in the form of acetate until four carbons remained, and that this 4-carbon chain was then oxidized to ketone bodies It became obvious that this theory was not adequate, when Jowett and Quastel (123) found that more than one ketone body was formed per molecule of fatty acid dissimilated The idea of a 4-carbon cleavage of the molecule was therefore suggested

MacKay *et al* (124) proposed, however, that the conversion was to acetate and that the acetate was then converted to acetone bodies Weinhouse, Medes and Floyd (125) have now presented convincing evidence of this mechanism of formation of acetone bodies from fatty acids When n-octanoate labeled by the incorporation of $C^{13}$ in the carboxyl group was incubated *in vitro* with liver slices the resulting acetoacetate contained the excess $C^{13}$ equally distributed between the carbonyl and carboxyl carbon atoms The only mechanism which satisfactorily explains these results is one which involves random coupling of $C_2$ units as illustrated in Reaction 33

(33) $CH_3\ (CH_2)_6\ C^{13}OOH \rightarrow 4CH_3\ C^{13}OOH \rightarrow 2CH_3\ C^{13}O\ CH_2\ C^{13}OOH$

The $C_2$ unit may not be acetic acid, but it is a likely possibility in view of the results of Swendseid *et al* (121) and Weinhouse *et al* (126) showing the $C^{13}$ acetate is converted to $C^{13}$-acetone bodies

It does not necessarily follow that none of the acetoacetic acid can be formed by direct conversion from a $C_4$ intermediate compound In fact, Medes *et al* (127) have demonstrated with carboxyl labeled butyrate that about 22 per cent was converted by direct oxidation to acetoacetate by the liver slices The acetoacetate formed by this conversion contains $C^{13}$ in the carboxyl group only, and for that reason the resulting acetoacetate contained more $C^{13}$ in the carboxyl group than in the carbonyl group The reaction may be as follows

(34) $CH_3\ CH_2\ CH_2\ COOH \rightarrow CH_3\ CO\ CH_2\ COOH \leftrightharpoons CH_3\ COOH$

In the case of octanoate, since the last 4 carbons did not contain any labeled carbon, the direct conversion could not be detected

These results, beyond their direct importance in contributing information concerning acetone body formation, give good evidence that $C_3$ components are formed from fatty acids

Bloch and Rittenberg (128) have made an important contribution to information on the formation of $C_2$ compounds from different compounds by using intact rats This was done by studying the rate of acetylation of phenylaminobutyric acid, when this compound and the test compound were included in the diet The compounds were labeled with deuterium and the amount of deuterium excreted as acetylphenylaminobutyric acid was determined The findings are interpreted on the assumption that acetic acid, or some closely related compound, is the direct acetylating agent and that the appearance of deuterio acetyl groups is proof of the intermediary formation of deuterio acetic acid from the isotopic test substance

Deuterio acetyl groups were excreted after administration of labeled ethanol, butyrate, alanine, n valerate and myristate, but not after propionate and 10, 11 dideutero undecylate The results are considered to be in complete agreement with the theory of $\beta$ oxidation to $C_2$ units The negative results with 10, 11-dideutero undecylic acid are believed to result because the 9, 10 and 11 carbon atoms are converted to propionate This compound did not yield an acetyl group The fact that propionate did not form an acetyl group is considered evidence that propionate is not oxidized to pyruvate (Step 23 of fig 1) If pyruvate was formed from propionate it is quite certain that the foreign amine would have been acetylated by the resulting pyruvate It is assumed that pyruvate, if formed, would acetylate since alanine is an effective acetylating agent and is believed to acetylate after its conversion to pyruvate Bloch and Rittenberg suggested therefore that the oxidation of propionate must be at the $\beta$ carbon and not at the $\alpha$ carbon in the conversion of propionate to glycogen It is interesting in relation to this point to note (Section II, p 214) that in the propionic acid fermentation the reverse reaction, conversion of pyruvate to propionate is not believed to occur by a direct reduction

Bloch and Rittenberg (112) have extended these studies of the acetylation of foreign amines by use of acetic acid labeled with deuterium in the methyl group and $C^{13}$ in the carboxyl group They have shown that there is no substantial change in the ratio of deuterium and $C^{13}$ during acetylation in the animal body This fact indicates that there is no loss of deuterium by exchange during the acetylation reaction It was necessary to establish this fact, since these authors were attempting to determine the amount of acetate formed in the body by measurement of the dilution of the deuterium in the acetylated amine as compared to that of the fed acetate The basis of the calculation is that the dilution is caused entirely by mixing of the administered acetate and acetate occurring in the animal body The above results showed that the dilution could not have been by loss of deuterium from acetate through exchange reactions After excluding this possibility it was necessary to assume that acetate is the only acetylating agent and that other compounds do not react with the amine and then become converted to an acetyl group Bloch and Rittenberg believe this

is true for aromatic amines, although they showed alanine will apparently acetylate phenylaminobutyric acid in a form other than acetate Their calculations indicate that from 15 to 20 mM of acetyl groups are formed per 100 grams of rat tissue per day

There are a number of possibilities for error in this determination Obviously any mechanism that removes the administered acetate without its coming to equilibrium with the acetyl groups will cause error This would be the result if acetylation involved formation of an intermediate and if acetate was oxidized or removed without coming to equilibrium with this intermediate The dilution by body acetyl groups would, in this circumstance, appear greater than is actually the case The opposite kind of error would be made if the $C_2$ compound, as formed in the cell, was oxidized at the site of formation without equilibrating with added acetate or with the $C_2$ compounds at the site of acetylation. In this case the calculated value of acetyl groups would be too low It should be borne in mind that acetate as such may be a dead end or side reaction, and that acetate may be converted to an intermediary $C_2$ component of the body but as such may not be the true intermediate of oxidation Nevertheless, the experiments of Rittenberg and Bloch are the first attempt to measure formation of $C_2$ compounds in the body and the results indicate there is a substantial amount of $C_2$ compounds formed in animal metabolism

It is interesting that Bloch and Rittenberg's results exclude the conversion of acetate to acetoacetate in the acetylation reaction If this reaction occurred there would be a loss of deuterium and, therefore, a change in the ratio of $C^{13}$ to deuterium in the acetylated amine

They suggest that the major part of the acetyl group arises by oxidation of fatty acids The mechanism of the reactions by which the fatty acids are oxidatively degraded to $C_2$ units has not been investigated extensively No intermediate products of $\beta$ oxidation have been isolated, likewise, it has not been possible to detect the lower fatty acids in oxidations by liver preparations (125), (129)

Lehninger (130) has demonstrated that adenosine triphosphate is required in oxidations by liver homogenates He suggests that an acyl phosphate is formed and that this phosphorylation activates the molecule for oxidation

c *Formation of intermediate compounds of the cycle from labeled acetate and acetoacetate* Aside from the evidence that fatty acids may be converted to $C_2$ compounds, there is additional evidence that fatty acids may be dissimilated by the tricarboxylic acid cycle, in that some of the intermediate steps of the cycle have been demonstrated The most convincing evidence has been supplied by Buchanan *et al* (85) using labeled acetoacetate and acetate and by Rittenberg and Bloch (89) and Weinhouse *et al* (88) using labeled acetate Prior to these experiments, there was ample evidence that the intermediate compounds of the cycle were rapidly fermented (79), (80), (42), (131), (107) by different tissues Only the evidence with labeled compounds can be considered in detail in this report

Buchanan *et al* (85) found that citrate, $\alpha$ ketoglutarate, succinate, fumarate, malate or oxalacetate stimulated the oxidation of acetoacate by homogenized guinea pig kidney cortex As a test of whether or not acetoacetate was oxidized by the tricarboxylic acid cycle the amount of conversion of $CH_3$ $C^{13}O$ $CH_3$ $C^{13}$ OOH to intermediates of the cycle was measured The isotopic acetoacetate was oxidized in mixture with succinate in one case and $\alpha$ ketoglutarate in another Fumarate was isolated from the experiment to which succinate was added and $\alpha$-ketoglutarate from the other experiment The isolated acids contained an excess of $C^{13}$ of such a magnitude as to indicate that the acetoacetate was oxidized *via* the tricarboxylic acid cycle

By degradation of the isolated acids the position of the $C^{13}$ was determined The excess $C^{13}$ was in the carboxyl groups of the fumarate. In the $\alpha$ ketoglutarate the carboxyl group adjacent to the keto group contained 0.24 per cent excess $C^{13}$ and the remainder of the molecule 0 59 per cent If all of the excess $C^{13}$ was in the two carboxyl groups of the $\alpha$ ketoglutarate, the excess $C^{13}$ in the second carboxyl group would be 2.36 per cent

When carboxyl labeled acetate was oxidized together with $\alpha$ ketoglutarate, succinate was formed which contained excess $C^{13}$ In a similar experiment by Weinhouse *et al* (88) with labeled acetate and rat kidney mince, the $\alpha$ ketoglutarate was isolated and degraded One-sixth of the total $C^{13}$ in the $\alpha$ ketoglutarate was found in the carboxyl adjacent to the carbonyl group, the remainder is presumed to be in the other carboxyl group

Weinhouse *et al.* (88) by a similar technique were unable to show the conversion of acetate to citrate when either citrate or aconitate was added together with $C^{13}$ acetate The significance of these results has been considered on p 224

The above results with $\alpha$ ketoglutarate and the dicarboxylic acids are in complete agreement with the proposals of the scheme of figure 1 The $\alpha$-ketoglutarate and succinate would, according to this scheme, be oxidized to oxalacetate by Steps 10, 11, 3, and 2 The resulting oxalacetate would unite with an acetyl group containing $C^{13}$ in the carboxyl group, the latter being formed from the acetoacetate by Step 18 Then by Steps 5, 6, 7, 8, 10, 11, $\alpha$-ketoglutarate and fumarate would be formed The $\alpha$-ketoglutarate would contain excess $C^{13}$ only in the carboxyl group distal to the carbonyl group and the fumarate would contain isotope only in the carboxyl groups If this $C^{13}$ fumarate re-entered the cycle and reacted with a $C^{13}$-acetyl group the resulting $\alpha$ ketoglutarate would contain excess $C^{13}$ in both carboxyl groups This may in part account for the occurrence of some isotope in the carboxyl adjacent to the keto groups in $\alpha$-ketoglutarate Partly the distribution in both carboxyls may result from the reversible side reaction to citrate, Step 21

The results obtained by Rittenberg and Bloch (89) are very similar and were obtained by feeding acetate labeled with $C^{13}$ in the carboxyl group to rats and mice The intermediates of the tricarboxylic acid cycle could not be isolated from the intact animal but glutamic and aspartic acids, which are in biological

equilibrium with α-ketoglutarate (Step 9, fig 1) and oxalacetate respectively, were isolated The carboxyl adjacent to the amino group in the glutamate, which was isolated from mouse liver, contained 0 096 per cent excess $C^{13}$ and the entire molecule contained 0 080 per cent If the excess $C^{13}$ was entirely in the two carboxyl groups, the carboxyl distal to the amino group would have contained 0 304 per cent excess $C^{13}$ The aspartate contained 0 038 per cent excess $C^{13}$ This compound was not degraded

It should be mentioned that the $C^{13}$ in the glutamate was greater than that in the respiratory carbon dioxide Therefore, the $C^{13}$ could not have been incorporated in the glutamate exclusively as carbon dioxide Presumably, however, some of the $C^{13}$ in the α carboxyl of the glutamate may have been incorporated by carbon dioxide fixation

It is evident that these results are almost identical with those obtained by Buchanan *et al* (85) and Weinhouse *et al* (88) The low concentration of $C^{13}$ found in the isolated compounds is to be expected because of the large dilution of acetate in the animal body and because the $C^{13}$-glutamate and asparate would be diluted by normal glutamate and asparate of the tissue The data are particularly important because they show that results obtained with the intact animal are adaptable to the tricarboxylic acid cycle

These results do not confirm Krebs and Eggleston's (132) and Weil-Malherbe's (133) contention that acetoacetate is not oxidized by the citrate cycle Krebs suggested that the increased production of citrate from oxalacetate and acetoacetate as compared to that from oxalacetate alone, was caused simply by the acetoacetate acting as a hydrogen acceptor and that the acetoacetate was not oxidized by the cycle

SUMMARY The experimental evidence for the tricarboxylic acid cycle has been critically examined and the point of greatest weakness in the evidence is considered to be that concerning the initial condensation reaction to form a tricarboxylic acid

The adaptability of the tricarboxylic acid cycle as an explanation of carbohydrate and fatty acid oxidation is discussed particularly in relation to recent observations made by isotope experiments The scheme satisfactorily accounts for the position of carbon dioxide fixed in pyruvate oxidation by pigeon liver Likewise, the formation of glycogen from acetate, propionate and butyrate can be explained by this mechanism, although for this explanation a slight modification of the scheme seems necessary

Oxidation of fatty acids via the tricarboxylic acid cycle is a likely possibility By assuming that the initial condensation reaction of the cycle involves a $C_2$ compound, an integrated picture of carbohydrate and fatty acid oxidation can be obtained The proposal that the initial condensation is a more complicated process than a $C_2$ and $C_4$ condensation is considered inadequately supported by experimental evidence For the sake of uniformity and simplicity, it is deemed advisable at present to picture the condensation to be with a $C_2$ component Fatty acid oxidation via the cycle can be explained since $C_2$ components are rapidly formed from fatty acids and also intermediate compounds of the cycle

are formed with incorporation of the carbon from the $C_2$ components in the predicted positions

## REFERENCES


(1) Evans E A Jr The Harvey Lecture Series 39 273 1944
(2) Werkman C H and H G Wood Advances in Enzymology New York Interscience Publishers 2 135, 1942
(3) Krebs H A Annual Review of Biochemistry Stanford University 12: 529, 1943
(4) Wood, H G and C H Werkman J Bact 30 332, 1935 Biochem J 30: 48, 1936
(5) Slade, H D H G Wood A O Nier, A Hemingway and C H Werkman J Biol Chem 143 133 1942
(6) Barker, H A, S Ruben and J V Beck Proc Natl Acad Sci 26 477 1940
(7) Foster, J W S F Carson, S Ruben and M D Kamen Proc Natl Acad Sci 27 500 1941
(8) Searle D S and L Reiner J Biol Chem 141 563 1941
(9) Van Niel, C B, J O Thomas S Ruben and M D Kamen Proc Natl Acad Sci 28: 157 1942
(10) Evans E A Jr and L Slotin J Biol Chem 141: 439 1941
(11) Wood, H G C H Werkman A. Hemingway and A O Nier J Biol Chem 142: 31 1942
(12) Solomon A K B Vennesland F W Klemperer J M Buchanan and A B Hastings J Biol Chem 140: 171 1941
(13) Lorber, V A Hemingway and A O Nier J Biol Chem 151 647, 1943
(14) Vogler K. G and W W Umbreit J Gen. Physiol 26: 157, 1942
(15) Ruben, S J Am Chem Soc 65 279 1943
(16) Lipman F and L C Tuttle J Biol Chem 158 505 1945
(17) Krampitz, L O H G Wood and C H Werkman J Biol Chem 147 243 1943
(18) Utter, M F F Lipmann and C H Werkman J Biol Chem 158: 521 1945
(19) Ochoa S J Biol Chem 159: 243 1945
(20) Wood H G and C H Werkman Biochem J 32 1262, 1938
(21) Wood H G, R. W Stone and C H Werkman Biochem J 31: 349, 1037
(22) Krampitz L O and C H Werkman Biochem J 35 595 1941
(23) Evans, E A. Jr B Vennesland and L Slotin J Biol Chem 147: 771 1943
(24) Wood H G, B Vennesland and E A Evans Jr J Biol Chem 159 153, 1945
(25) Utter M F and H G Wood J Biol Chem 160 375 1945
(26) Gunsalus I C W D Bellamy and W W Umbreit J Biol Chem 155 685 1944
(27) Kalnitsky, G and C H Werkman Arch Biochem 4 25 1944
(28) Straub, F B Ztschr Physiol Chem 244: 117 1936
(29) Carson S F, S Ruben M D Kamen and J W Foster Proc Natl Acad Sci 27 475 1941
(30) Brown, R W, H G Wood and C H Werkman Arch Biochem 5: 423 1944
(31) Utter, M F and C H Werkman Arch Biochem 2: 491 1943 5: 413, 1944
(32) Lipmann F Cold Springs Harbor Symposia Quant Biol 7 248 1939
(33) Lipmann F Advances in Enzymology New York Interscience Publishers 1: 99 1941
(34) Silverman, M and C H Werkman Proc Soc Exper Biol Med 43 777 1940 J Biol Chem 138: 35 1941
(35) Kalnitsky G and C H Werkman Arch Biochem 2 113 1943
(36) Lipmann F and L C Tuttle J Biol Chem 154: 725 1944
(37) Lipmann, F J Biol Chem 155 55, 1944
(38) Koepsell, H J and M J Johnson J Biol Chem 145: 379, 1942
(39) Koepsell, H J M J Johnson and J S Meek J Biol Chem 154: 535, 1944

(40) WILSON, J , L O KRAMPITZ AND C H WERKMAN Private communication
(41) LIPMANN, F Proc Div Biol Chem 46B 108th Meeting American Chemical Society, 1944
(42) KREBS, H A Advances in Enzymology New York, Interscience Publishers, 3 191, 1943
(43) OCHOA, S AND E WEISZ-TABORI J Biol Chem 159 245, 1945
(44) MARTIUS, C Ztschr Physiol Chem 247 104, 1937
(45) BARKER, H A , S RUBEN AND M D KAMEN Proc Natl Acad Sci 26 426, 1940
(46) BARKER, H A Arch Mikrobiol 7 404, 1936
(47) BARKER, H A J Biol Chem 137 153, 1941
(48) KREBS, H A AND K HENSELEIT Ztschr Physiol Chem 210 33, 1932
(49) RITTENBERG, D AND H WAELSCH J Biol Chem 136 799, 1940
(50) EVANS, E A , JR AND L SLOTIN J Biol Chem 136 805, 1940
(51) WOODS, D D Biochem J 30 515, 1936
(52) WOOD, H G , C H WERKMAN, A HEMINGWAY AND A O NIER J Biol Chem 139 365, 1941
(53) KALNITSKY, G , H G WOOD AND C H WERKMAN Arch Biochem 2 269, 1943
(54) FONTAINE, F E , W H PETERSON, E MCCOY, M J JOHNSON AND G J RITTER J Bact 43 701, 1942
(55) BARKER, H A Proc Natl Acad Sci 30 88, 1944
(56) SLADE, H D AND C H WERKMAN Arch Biochem 2 97, 1943
(57) THUNBERG, T Skand Arch Physiol 40 1, 1920
(58) CARSON, S F AND S RUBEN Proc Natl Acad Sci 26 422, 1940
(59) WOOD, H G , C H WERKMAN, A HEMINGWAY AND A O NIER Proc Soc Exper Biol and Med 46 313, 1941
(60) CARSON, S F , J W FOSTER, S RUBEN AND H A BARKER Proc Natl Acad Sci 27 229, 1941
(61) BARKER, H A AND F LIPMANN Arch Biochem 4 361, 1944
(62) CHAIX-AUDEMARD, P Sur les relations existant entre respiration et fermentation chez propionibacterium pentosaceum Lyon, 1940
(63) NISHINA, Y , S ENDO AND H NAKAYAMA Sci Papers Inst Phys Chem Research, Tokyo 38 341, 1941
(64) EVANS, E A , JR AND L SLOTIN J Biol Chem 136 301, 1940
(65) WOOD, H G , C H WERKMAN, A HEMINGWAY AND A O NIER J Biol Chem 139 483, 1941
(66) EVANS, E A , JR AND L SLOTIN J Biol Chem 141 439, 1941
(67) STOTZ, E Advances in Enzymology New York, Interscience Publishers 5 153, 1945
(68) VENNESLAND, B , A K SOLOMON, J M BUCHANAN AND A B HASTINGS J Biol Chem 142 379, 1942
(69) BUCHANAN, J M , A B HASTINGS AND F B NESBETT J Biol Chem 145 715, 1942
(70) CONANT, J B , R C CRAMER, A B HASTINGS, F W KLEMPERER, A K SOLOMON AND B VENNESLAND J Biol Chem 137 557, 1941
(71) VENNESLAND, B , A K SOLOMON, J M BUCHANAN, R D CRAMER AND A B HASTINGS J Biol Chem 142 371, 1942
(72) BUCHANAN, J M , A B HASTINGS AND F B NESBETT J Biol Chem 150 413, 1943
(73) MEYERHOF, O , P OHLMEYER, W GENTNER AND W MAIER-LEIBNITZ Biochem Ztschr 298 396, 1938
(74) LARDY, H A AND J A ZIEGLER J Biol Chem 159 343, 1945
(75) WOOD, H G , N LIFSON AND V LORBER J Biol Chem 159 475, 1945
(76) LORBER, V , N LIFSON AND H G WOOD J Biol Chem 161 411, 1945, J Biol Chem , submitted

(77) Swenseid, M E The use of the stable isotope, heavy carbon in study of intermediary metabolism Ph.D Thesis University of Minnesota
(78) Krebs, H A and W A Johnson Enzymologia 4: 148 1937
(79) Krebs, H A and L V Eggleston Biochem J 34 442 1940
(80) Evans E A Jr Biochem J 34: 829 1940
(81) Krebs, H A and L V Eggleston Biochem J 34 1380 1940
(82) Smith D H Biochem J 34 1046 1940
(83) Breusch F L Science 97: 490 1943
(84) Wieland, H and C Rosenthal Annalen 554 241 1943
(85) Buchanan J M W Sakami S Gurin and D W Wilson J Biol Chem 157 747 1945, 159 695 1945
(86) Hunter E and L F Leloir J Biol Chem 159: 295 1945
(87) Lynen, F Annalen 554: 40 1943
(88) Weinhouse, S, G Medes N F Floyd and L Noda J Biol Chem 161 745 1945
(89) Rittenberg D and K Bloch J Biol Chem 157 749 1945
(90) Krebs H A Nature 138 288 1936
(91) Szent Györgyi A V Studies on biological oxidations Stechert New York 1937
(92) Krebs H A Biochem J 37 IX 1943
(93) Ochoa S J Biol Chem 151 493 1943
(94) Ochoa S J Biol Chem 149 577, 1943 155 87 1944
(95) Ochoa S J Biol Chem 138 751, 1941
(96) Kalckar H M Biol Revs Cambridge Phil Soc 17 28 1942 Muñoz, J M and L F Leloir J Biol Chem 147 355 1943
(97) Krebs H A Biochem J 34 775 1940
(98) Kalckar H M Biochem J 33: 631 1939
(99) Leloir L F and J M Muñoz J Biol Chem 153: 53 1944
(100) Blixenkrone Möller N Ztschr Physiol Chem 252 137 1938
(101) Wood H G N Lifson and V Lorber Unpublished results
(102) Schoenheimer, R and D Rittenberg Physiol Rev 20: 218 1940
(103) Deuel H J, Jr C Johnston M G Morehouse H S Rollman and R. J Winzler J Biol Chem 157 135 1945
(104) Deuel H J, Jr J S Butts H Blunden C H Cutler and L Knott J Biol Chem 117: 119 1937
(105) Ponsford A P and I Smedley MacLean Biochem J 26 1340 1932
(106) Stadie, W C Physiol Rev 25 395 1945
(107) Lynen F Annalen 552: 272 1942
(108) Sonderhoff R and H Thomas Annalen 530: 195 1937
(109) Wieland, H and R. Gottfried Annalen 548 255 1941
(110) Lehninger A L J Biol Chem 161 413 1945
(111) Martius C Ztschr Physiol Chem 279: 96 1943
(112) Bloch, K and D Rittenberg J Biol Chem 159 45 1945
(113) Bloch K. and D Rittenberg J Biol Chem 143: 297 1942
(114) Rittenberg D and K Bloch J Biol Chem 154 311, 1944 J Biol Chem 161 417, 1945
(115) Olsen N S, A Hemingway and A O Nier J Biol Chem 148: 611 1943
(116) Shemin D and D Rittenberg J Biol Chem 159: 567 1945
(117) Bloch K and R Schoenheimer J Biol Chem 138: 167 1941
(118) Knoop F Beitr Chem Physiol u Path 6 150 1904
(119) Stetten DeW Jr and R. Schoenheimer J Biol Chem 133: 329 1940
(120) Schoenheimer R. and D Rittenberg J Biol Chem 120: 155 1937
(121) Swendseid M E R H Barnes A Hemingway and A. O Nier J Biol Chem. 142 47 1942
(122) Wood H G, R W Brown and C H Werkman Arch Biochem 6: 243 1945
(123) Jowett M and J H Quastel Biochem J 29: 2159 1935

(124) MacKay, E M , R H Barnes, H O Carne and A N Wick J Biol Chem. 135 157, 1940
(125) Weinhouse, S , G Medes and N F Floyd J Biol Chem 155 143, 1944
(126) Weinhouse, S , G Medes and N F Floyd J Biol Chem 158 411, 1945
(127) Medes, G , S Weinhouse and N F Floyd J Biol Chem 157 35, 1945
(128) Bloch, K and D Rittenberg J Biol Chem 155 243, 1944
(129) Stadie, W C , J A Zapp, Jr and F D W Lukens J Biol Chem 137 75, 1941.
(130) Lehninger, A L J Biol Chem 157 368, 1945
(131) Evans, E A , Jr Bull Johns Hopkins Hospital 59 225, 1941
(132) Krebs, H and L Eggleston Nature 154 209, 1944
(133) Weil-Malherbe, H Nature 153 435, 1944
(134) Barker, H A and M D Kamen Proc Natl Acad Sci 31 219, 1945
(135) Barker, H A , M D Kamen and V Haas Proc Natl Acad Sci 31 355, 1945
(136) Weiringa, K T Leeuwenhock 3 1, 1936, 6 251, 1940
(137) Barker, H A , M D Kamen and B T Bornstein Proc Natl Acad Sci. 31. 373, 1945
(138) Moulder, J W , B Vennesland and E A Evans, Jr J Biol Chem 160 305, 1945
(139) Evans, E A , Jr A symposium on respiratory enzymes, Madison, Wis , 197, 1942
(140) Kalckar, H M , J Dehlinger and A Mehler J Biol Chem 154 275, 1944
(141) Furchgott, R F and E Shorr J Biol Chem 151 65, 1943
(142) Sacks, J Am J Physiol 129 227, 1940

# THE EFFECT OF BODY TEMPERATURE ON DRUG ACTION

FREDERICK A FUHRMAN

*Department of Physiology Stanford University, California*

The action of drugs in animals is influenced by body temperature as well as by the more commonly recognised variables such as body weight, sex and species. In poikilotherms, the temperature of the body is dependent upon that of the environment and may be varied over a range of about 35°C In homeotherms, the fluctations in body temperature are so slight that the influence of temperature upon pharmacological action is difficult to measure in completely normal animals, and is usually neglected It is only when the body temperature deviates markedly from normal that the effect of such changes becomes important It has only recently been appreciated that human beings can recover from body temperatures as low as 23°C (Talbott, 1941), although this was known much earlier (Reincke, 1875) Body temperature actually can be varied over a range of about 20°C in man and about 35°C in some other non hibernating mammals Since such ranges of temperature are possible, the influence of body temperature upon drug action becomes of considerable importance in homeotherms as well as in poikilotherms

The effect of temperature upon drug action has been investigated under three distinctly different circumstances (a) in homeotherms at varied levels of environmental temperature, (b) in both homeotherms and poikilotherms at varied body temperatures, and (c) in isolated organs and tissues at graded temperatue levels. This review includes chiefly the alterations in drug action in vertebrates resulting from changes in body temperature Results obtained at varied environmental temperatues and on isolated preparations are included in some cases to supplement these observations.

One approach to the problem of the effect of temperatue upon drug action is the determination of the extent to which temperature coefficients may be modified by the presence of drugs. Some examples of such results, obtained chiefly on invertebrates, are cited by Bělehrádek (1935) and Crozier (1924) The effect of the following substances on the temperature coefficient of the heart beat of the frog has been reported epinephrine, tyramine, ethyl alcohol, guanidine, atropine, caffeine, pilocarpine, camphor and inorganic ions (cf Bělehrádek, 1932) For methods of calculation and interpretation of temperature coefficients the reviews by Prabaum (1923), Kanitz (1915, 1925), Bělehrádek (1935), Hoagland (1936), Sizer (1943) and van Rysselberghe (1944) may be consulted Of particular interest are the recent studies of temperature and the action of narcotics and other drugs on bacterial luminescence (F H Johnson et al , 1942, 1943, McElroy , 1943), and the application to these data of the theory of absolute reaction rates (cf Glasstone et al , 1941, Eyring and Magee, 1942)

It has not been possible to include all papers in the literature dealing with temperature and drug action In particular, many of the older references cited

by Brunton (1885), Richet (1889), Saint-Hilaire (1893), Langlois and Richet (1895) and Matisse (1919) have been omitted The effects of environmental temperature on vitamin requirements and the effect of temperature on bacteri cidal action are not included

PHYSIOLOGICAL EFFECTS OF TEMPERATURE The effects of temperature on drug action, particularly in homeotherms, cannot properly be evaluated without first considering the effect of changes in body temperature upon physiological processes The alteration in pharmacodynamic response resulting from lowering of body temperature, for example, is not due entirely to the simple effect of temperature upon the rate of chemical reactions, but is often greatly modified by circulatory, nervous or metabolic changes resulting from the decrease in temperature For this reason, certain temperature effects, uncomplicated by the action of drugs, and ranges of body temperature compatible with vertebrate life, are discussed below

*Poikilotherms* Under laboratory conditions the body temperature of poikilotherms differs little from that of the environment, being, in aquatic animals, less than 1°C warmer than that of the water (D L Gunn, 1942) Amphibia, removed from the water, lose heat by evaporation and therefore have a body temperature lower than that of the air (except at 100 per cent humidity) (Adolph, 1932, Hall and Root, 1930) For practical purposes the body temperature of frogs in cages filled with sufficient water to cover the animals may be considered to be identical with that of the water This situation does not exist, however, if the frogs are permitted to emerge from the water into a room at low relative humidity

The metabolic rate of poikilotherms rises with increase in environmental temperature, the relationship is, however, non-linear (cf Benedict, 1932, O'Connor and O'Donovan, 1942)

The highest body temperature tolerated by a given animal is greatly influenced by the time of exposure (cf Bělehrádek, 1935) Unfortunately few authors have provided sufficient data to evaluate the importance of the time factor Some frogs (*R pipiens*) in a given group die within 24 hours in water at 33°C (Chen et al , 1943a), and death occurs more rapidly at higher temperatures (Tigerstedt 1910, Kanitz, 1925) Many fish do not tolerate temperatures above 30°C (Britton, 1924) Frogs and certain fish survive cooling to temperatures slightly below 0°C (Cameron and Brownlee, 1914, Britton, 1924) The cause of death at both high and low temperatures is discussed by Heilbrunn (1943)

*Homeotherms* Large homeotherms maintain a fairly constant body temperature at environmental temperatures from below 0°C to above 40°C The body temperature of mice and other small homeotherms, however, is in part determined by the temperature of the environment (Nielsen, 1938, Goldfeder, 1941, Chen et al , 1943b) For example, mice placed on a wire screen platform in individual glass cages at 15°C showed a mean decrease in deep rectal temperature of 2 36°C in one hour (G J Fuhrman, 1943) In mice, increased body temperature results from exposure to an environmental temperature of about 35°C or above For this reason attempts to investigate variations in drug

action in small animals at varied environmental temperatures may be complicated at both high and low extremes by changes in body temperature of the animals. In many studies at different environmental temperatures, body temperatures have not been reported Anesthesia (Hemingway, 1940) and curare (Krogh 1916) impair the temperature regulation of homeotherms to such an extent that the body temperature may then be manipulated by cooling or warming Hibernating mammals appear to have a "labile" body temperature in the non hibernating state (Benedict and Lee, 1938)

Acute hyperthermia in mammals results in increased metabolic rate, increased respiratory and heart rate and decrease in total peripheral resistance which may be more than compensated for by increased cardiac output (cf Wiggers and Orias, 1932, Bazett, 1938) Death may occur from either respiratory or cardiac failure in the temperature range 40° to 45°C (Kanitz, 1925, Marsh, 1930) The lethal level of hyperthermia in the fowl is about 47°C (Randall, 1943)

Progressive general hypothermia (36° to 30°C ) in mammals results at first in increased heat production (from shivering and muscle rigidity) and hyperreflexia (Dill and Forbes, 1941, Fay and Smith, 1941 Grosse Brockhoff and Schoedel, 1943a) At lower temperatures metabolic rate, heart rate, respiratory rate, cardiac output and arterial pressure all decrease Reflexes are progressively abolished (Hamilton, 1937, cf Libet et al , 1940) Lethal levels of hypothermia vary from about 20°C in the dog (Grosse-Brockhoff and Schoedel, 1943b) to 13-16°C in the rat (Crismon, 1944) and 10°C in the rabbit (Ariel et al , 1943) Lower levels of hypothermia may perhaps be tolerated if some form of treatment precedes cooling, respiratory movements and cardiac activity were observed between 5° and 6°C in fasted rats (Crismon and Fuhrman, 1946) The mechanism of death from hypothermia has been discussed by Grosse-Brockhoff and Schoedel (1943b) and by Crismon (1944) Hibernating mammals tolerate body temperatures as low as 0° to 3°C (G E Johnson, 1931, Benedict and Lee, 1938)

*Isolated tissues* Isolated vertebrate tissues survive over the approximate temperature range 0° to 42°C if oxygen consumption and lactic acid production be taken as the criteria of survival (Fuhrman and Field, 1943, 1945, Field et al , 1944 G J Fuhrman, unpublished) The capacity for oxygen consumption of isolated frog muscle is impaired by a temperature approximately that necessary to produce heat paralysis in the intact animal (Brachet and Bremer, 1941, Feitelberg and Pick, 1942) Thermal reflex block in spinal centers of a frog nerve-muscle preparation occurred above 29°C and below 14°C (Ozorio de Almeida, 1943)

General comparisons drug action in homeotherms and in poikilotherms Inasmuch as there is a body temperature difference of almost 20°C between mammals and frogs at room temperature, a comparison of pharmacodynamic effects in the two groups of animals yields data which depend in part upon the temperature difference Such data are, in general, difficult to evaluate because of species differences.

*Colchicine* Poisoning from seeds and galenical preparations of meadow saffron (*Colchicum autumnale*), and from eating the leaves as salad, led Jacobj (1890) to

undertake a more complete pharmacological investigation of the active alkaloid colchicine, than had previously been made He found that colchicine was fa more toxic in mammals than in frogs Earlier workers (cited by Jacobj) ha found lethal doses to be extremely variable in both amphibia and mamma because of the use of impure and partially oxidized preparations Jacobj pr pared an oxidation product of colchicine, oxydicolchicine, and found that th action of this substance was qualitatively and quantitatively similar in bot mammals and frogs He concluded that colchicine itself is relatively non-toxi and that it is oxidized in the body to the toxic oxydicolchicine In the frog, low temperatures, oxidation does not occur rapidly enough to produce tox symptoms before excretion has occurred Führer (1910) and Sanno (1911) four that the toxicity of colchicine in frogs (*R esculenta*) was 400 to 500 times as gre at 32°C as at room temperature (about 20°C ) Frogs injected with colchicin and maintained at room temperature showed no toxic symptoms during sever days, but died upon being warmed to 30–32°C

In mammals the symptoms of colchicine poisoning are not observed until 3 6 hours after injection (Jacobj, 1890), and this latent period is not marked shortened by increasing the dose In frogs, at 20°C death occurs after 14 da and at 30°C after 48 hrs (Führer and Breipohl, 1933)

In later experiments the fatal dose for frogs at 20°C was found to be 1 3 mg per gm , while in mice it was 0 003 mgm per gm (Führer, 1932) The fat dose for frogs at 30°C was found to be 0 003 mgm per gm (Führer and Br pohl, 1933) The striking parallelism between the increase in toxicity in war compared to cold frogs, and in mice compared to cold frogs, suggests that t temperature of the body is the chief factor influencing toxicity The more rap conversion of colchicine to the toxic oxidation product appears to be a satisfa tory explanation for the results obtained

*Mussel poison* Frogs are much more resistant than are mammals toward t paralytic shell-fish poison from mussels (*Mytilus Californianus*) (Prinzmet Sommer and Leake, 1932)

*Miscellaneous substances* Fuhner (1932) made an extensive series of compa sons of fatal doses of various drugs in frogs and mice The temperature diff ence between the two groups was about 17°C He found that methylguanidi synephrine, phenol, caffeine, resorcinol and nupercaine were more toxic in t frog than in the mouse, the ratio of fatal doses (frog/mouse) ranged from 0.3 0 8 Histamine, procaine, chloral hydrate, pyrocatechin, hydroquinone a camphor were approximately equal in toxicity in the two groups of anima Acetylcholine, aminopyrine, picrotoxin and sodium cyanide were more toxic the mouse than in the frog, with ratios ranging from 2 to 6 5 Epinephrine w about 60 times as toxic in the mouse as in the frog

A number of drugs produce central nervous system stimulation in home therms, but produce depression in frogs Sodium 1,3-dimethyl-butyl-eth barbiturate produces convulsions in homeotherms (mice, rats, guinea pigs, ra bits, cats, dogs, monkeys) but only depression in poikilotherms (frogs and toa (Swanson and Chen, 1939, Knoefel, 1945) Sodium 1-methyl-allyl-isobut

thiobarbiturate acts in a similar manner (Swanson, Fry and Reagan, 1945) Cocaine (J A. Gunn, 1937) and harmine (Chen and Chen, 1932) likewise are convulsants in homeotherms and depressants in poikilotherms Harmine is about twice as toxic in mice at an environmental temperature of 40°C as at 20°C (Chen et al., 1943b)

EFFECTS OF DRUGS DURING HIBERNATION The body temperature of mammals during hibernation falls to a level only slightly above that of the environment Differences observed in the effect of drugs in animals during hibernation and the effects in animals at body temperatures of 37–39°C may be in part accounted for on the basis of the difference in temperature Such a comparison is, however, complicated by physiological changes occurring during hibernation, e.g., acidosis (Stormont et al , 1939)

In 1899 Koennick reported results of the subcutaneous administration of strychnine, picrotoxin, muscarine, pilocarpine, caffeine and apomorphine to a few hibernating bats (*Nannugo Pipistrellus*) caught in the cellar of the Marburg castle At an environmental temperature of 6–7°C the effects were somewhat slower in appearing than in non-hibernating animals, but the actions were qualitatively similar Koennick reported that hibernating bats tolerated larger doses of tetanus toxin than did bats which were not hibernating His data for the other substances are not sufficient to permit quantitative comparison with animals at normal body temperatures Hausmann (1906), using larger numbers of bats (*Vesperugo noctula*) found that colchicine, saponin, tannin and abrin produced death much more rapidly in bats which were active than in hibernating bats. Larger doses of colchicine were tolerated by the hibernating animals Marmots injected with tetanus toxin showed no symptoms of tetanus while hibernating, but developed tetanus upon awakening (Billinger, 1896)

Marmots during hibernation were about as sensitive as non hibernating animals to the effects of cobra venom, diphtheria and tetanus toxin and inoculation with parasites, the effects were slower in developing in the hibernating animals (Blanchard, 1903)

Recently Pfeiffer, Foster and Slight (1939) carried out a more extensive study of the effects of a number of analeptic drugs on the thirteen lined ground squirrel (*Citellus tridecemlineatus*) Active animals were at a body temperature of 32–40°C , while hibernating the body temperature of the squirrels was 4–10°C Lethal and convulsant doses were determined in the awake and hibernating animals for ephedrine, neosynephrine, epinephrine, benzedrine, cocaine, picrotoxin, metrazol, coramine, caffeine with sodium benzoate and strychnine It was found that caffeine and strychnine were 8 and 10 times, respectively, more toxic in the hibernating animals than in active ones, probably as a result of the high blood $pCO_2$ in hibernation. Asphyxia renders normal animals more susceptible to strychnine convulsions Epinephrine produced convulsions in hibernating animals only Clonic convulsions, produced by coramine for example, were well tolerated by the hibernating animals, and persisted for 2–3 days Convulsions produced by strychnine were poorly tolerated.

The effect of various drugs in awakening animals from the hibernating state,

and their relative efficiency (cf Pfeiffer et al , 1939) are not within the scope of the present review

DIGITALIS AND RELATED SUBSTANCES *Poikilotherms* Ever since attempts were made early in this century to use frogs for the bioassay of digitalis, various investigators have recognized that season and temperature are among the factors influencing the sensitivity of frogs to the cardiac action of the digitalis glycosides In the early work, part of the "seasonal variation" was undoubtedly due to temperature, but later results indicate a seasonal variation independent of assay temperature Focke (1907) was one of the first actually to demonstrate the influence of temperature upon the sensitivity of frogs He found that by warming frogs in winter he could increase their sensitivity to about that of summer frogs In the next few years Edmunds and Hale (1908), Hale (1911), Ginzberg and Hohlberg (1913), Vanderkleed and Pittinger (1913) and Gottlieb (1914) all recognized the importance of temperature in the frog method of bioassay of digitalis.

Baker (1912), using the 1 hour frog method (cf Munch, 1931 for this and other methods of bioassay), found that from 10° to 30°C there was a progressive decrease in the minimum systolic dose (MSD) of both ouabain and digitalis Haskell (1914), using Houghton's lethal method, obtained similar results Both found that the difference in toxicity of ouabain with change in temperature was greater than that of digitalis The MSD of digitoxin, determined by the 1 hour frog method, was found to be 13, 9 and 8 mgm per kgm at 10°C , 20°C and 30°C respectively (Roth, 1916) Weese (1936, p 20) gives lethal doses for frogs of digitoxin, gitalin, ouabain and strophanthin-k, determined by Houghton's lethal method, at 15°, 20° and 25°C which also clearly demonstrate increased toxicity at higher temperatures Goldfish died more rapidly in warm digitalis solutions than in cold ones (Pittinger and Vanderkleed, 1915) Sollmann et al (1915) determined the MSD of ouabain by the 1 hour frog method over the range 3° to 30°C , and found that the toxicity increases markedly with increase in temperature At 3–4°C the MSD was 26 1 mgm per kgm , while at 26°C it was only 0 3 mgm per kgm The increase in toxicity per degree of temperature increase was much greater at the lower than at the higher temperatures Hirschfelder et al (1920) extended these observations on frogs as high as 36°C and found increased toxicity of digitalis from 30° to 36°C Higher temperatures cannot be studied using frogs because of excessive mortality of controls at these temperatures Sollmann et al (1915), Roth (1916) and Defandorf (1933) recognized that differences in rate of absorption at different temperatures complicated the interpretation of the results obtained on intact frogs Smith and McClosky (1925) circumvented the complicating effects of temperature upon absorption rate by intravenous injection of digitalis in frogs They found that at 15°, 20°, 25°, 30° and 37°C (body temperature) the MSD for an international digitalis leaf powder was 740, 400, 300, 250 and 80 mgm per kgm respectively Thus, although rate of absorption may vary with temperature, a large part of the difference found is due to a more direct effect of temperature upon the action of digitalis

Curves relating temperature to MSD for ouabain (Baker, 1912, Sollmann et al ,

1915) and for digitalis (Smith and McClosky, 1925) are similar in shape On the basis of temperature effects there is therefore no reason to believe that the fundamental action of these two substances is different, as had been inferred from a comparison of their effects following injection into the lymph sac (cf Smith and McClosky, 1925) Differences in rate of absorption of the two drugs apparently led to the differences obtained by Baker (1912)

Chen et al (1943a) have recently determined the effect of temperature upon the $LD_{10}$ of two other digitalis-like substances cymarin, the active glycoside from Canadian Hemp (*Apocynum*), and coumingine hydrochloride, an alkaloid from *Erythrophloeum couminga* They were studied in *R pipiens* over the temperature range 13° to 33°C Coumingine hydrochloride was approximately 5 times as potent at 33°C as at 13°C , while cymarin was about twice as active at the higher temperature

*Isolated heart.* Trendelenburg (1909) noted that with strophanthin k and antiarin (from *Antiaris toxicaria*, the Upas tree of Java) the time required to produce systolic standstill of the isolated (Straub) heart increased as the temperature was lowered This observation has been confirmed and the data extended to other drugs of the digitalis series. This work was done chiefly by Weissäcker (1913) and Sollmann et al (1915) using ouabain, Fischer (1928) using digitoxin and gitoxigenin, Gander (1932) using gitalin and its aglycone gitaligenin

H. Fischer (cf Weese, 1936, p 127) in a series of studies of digitalis-like substances on the isolated frog heart, separated the binding of digitalis by the heart muscle into several phases I—membrane phase, II—fixation phase, III—action phase The influence of temperature upon phases II and III was studied in order to determine whether or not these were chemical in nature Fixation occurs more rapidly at higher temperatures (v Issekutz, 1914, Sollmann et al , 1915) The $Q_{10}$ for the process of fixation (phase II) lies between 2.3 and 3 8 for digitoxin (Fischer, 1928) and gitalin (Gander, 1932) The $Q_{10}$ for the rate of action (phase III) is of the same general magnitude (2–3) for digitoxin, gitoxigenin (Fischer, 1928), gitalin and gitaligenin (Gander, 1932) On the basis of a $Q_{10}$ of 2 to 3 the processes of fixation and of action in producing systolic standstill are therefore believed to be chemical, rather than physical, in nature

The $Q_{10}$ of the time required for the production of systolic standstill of the frog heart by ouabain varied from 6 1 to 17 (Sollmann et al , 1915) The activity was dependent upon both temperature and heart rate, the high $Q_{10}$ values were accounted for on the basis of a summation of the effect of temperature upon both processes. Weissäcker (1913), however, maintained the heart rate constant, and obtained data showing that the rate of action of digitalis increases with increase in temperature Fischer (1928) found the action of temperature in increasing the rate of action of digitoxin largely independent of the effect of temperature on the heart rate and amplitude The effect of temperature on the rate of fixation and of action was not dependent upon the calcium content of the Ringer solution (Fischer, 1928)

The concentration of drug necessary to produce systolic standstill *in a given time* also decreases with increase in temperature for strophanthin k (Trendelen

burg, 1909), ouabain (Sollmann et al , 1915), digitoxin, gitoxigenin (Fisch 1928), *gitalin and gitaligenin (Gander, 1932)* Likewise, the lowest concen tion of drug which will produce systolic standstill (Grenzkoncentration) is lo at higher temperatures, not only in the perfused frog heart, but also when st of frog heart sinus are used (Mansfeld and Horn, 1928) On the embry chick heart Paff and Johnson (1938) found that a given concentration of oual produced A-V block more rapidly at 38°C than at 28° or 20°C Block produ with 1 600,000 ouabain at 38°C could be made to disappear by reducing temperature to about 20°C

In the isolated perfused rabbit heart, ouabain produced complete arrest n rapidly at higher temperatures (J W C Gunn, 1914) At a given tempera and a given strength of ouabain solution, differences in rate of action were tained by varying the rate of coronary perfusion Gunn believed variatio rate of blood flow to be responsible for the temperature effects However, effect of temperature was still evident in the figures given when the rate of c nary perfusion was constant 1 500,000 ouabain, 218 cc stopped the heart i min at 30°C , 220 cc stopped the heart in 21 min at 40°C

In general the results obtained on the isolated perfused heart are in agreem with those obtained on intact frogs In both types of preparations the inten of action of cardiac glycosides increases with increase in temperature

*Homeotherms* The effect of temperature upon the action of digitalis homeotherms has been extensively studied only during hyperthermia In n and in some experimental work where fever was produced by toxins or infecti the effect of temperature may be complicated by the action of toxins on the h (cf Weese, 1936, p 233, McGuigan, 1938)

Jamieson (1915) reported the same lethal dose of ouabain in cats infected v pneumonia as in normal animals, but the body temperature rise in the infec animals was usually less than 1°C Herzog and Schwartz (1930) found that lethal dose of strophanthin was greater in cats and rabbits with fever than normal animals The isolated heart of the animals with fever was stopped v approximately the same dose required to stop the heart of normal animals, so t the authors concluded that the lower sensitivity of the intact animals with fe was of extra-cardiac origin Weese (1932) however, reported a decreased se tivity of the isolated heart to strophanthin only when the heart of an animal w fever was perfused with blood from animals with fever He attributed the dif ence to cardiac and humoral changes resulting from the fever

Data in which the results are not complicated by the presence of toxins do confirm the results of Herzog and Schwartz The lethal doses of tincture digitalis, in anesthetized cats warmed by immersion in water, were found to *0 94, 0 78, 0 59 and 0 375 cc per kgm at body temperatures of 38, 41, 42* and 43 respectively (Hirschfelder et al , 1920) McGuigan (1938) administered digit in divided doses to dogs under barbiturate anesthesia The MLD in cont animals was 123 mgm per kgm , while in animals in which hyperthermia (aver increase in temperature = 3 25°C ) was induced by the administration of d trophenol, the MLD was about 73 per cent of the control In further exp

ments, in which hyperthermia was induced by diathermy, the MLD at 40°, 41°, 42°, 43° and 44°C amounted to 82, 81, 66, 61 and 50 per cent of the control respectively Slowing of the heart rate was produced by digitalis during hyperthermia

The therapeutic implications of these experiments are evident The tachycardia in fever is chiefly the result of the direct effect of temperature upon the heart muscle Although slowing of the heart may be produced by digitalis, this may be obscured by the speeding of the heart resulting from rise in temperature Larger doses of digitalis cannot, however, be tolerated, since for each 1°C rise in temperature the toxicity of digitalis is increased 10 to 15 per cent Both Hirschfelder et al (1920) and McGuigan (1938) caution against the use of large doses of digitalis in fever

*Comparisons between homeotherms and poikilotherms* The very great difference between the fatal dose of digitalis glycosides and pharmacologically related substances in the frog and in the cat or dog is evident from the foregoing discussion McGuigan (1938) cites fatal doses of digitalis in the dog as 100 mgm per kgm and in the frog as 600 mgm per kgm , and attributes much of this difference to a difference in body temperature Führer (1931) reported that ouabain and cymarin were more toxic in the frog than in the mouse, but this discrepancy is to be accounted for on the basis of the well known resistance of mice (and rats) to digitalis-like substances (cf Chen and Rose, 1942) Cats, rabbits and guinea pigs (Chen and Rose, 1942) are much more susceptible to the action of cymarin, ouabain and coumingine hydrochloride than are Leopard frogs (*R pipiens*) or tree frogs (*Hyla cinerea cinerea*) (Blair, Hargreaves and Chen, 1940) Since the frogs were studied at 20°C , there was a body temperature difference of about 17°C between the homeotherms and poikilotherms. This may account for a large part of the difference in sensitivity Slow absorption from the lymph sac in frogs may also be important Smith and McClosky (1925) determined the MLD of digitalis in the cat to be 90 mgm. per kgm , by intravenous administration to frogs (where rate of absorption is not a factor) the MLD was found to be 80 mgm per kgm. at 37°C It would be preferable to make such a comparison at a body temperature of about 30°C for both animals, since frogs tolerate 37°C for only brief periods. The close agreement between the two fatal doses at 37°C suggests that temperature is chiefly responsible for the differences in fatal dose found between homeotherms at 37°C and poikilotherms at about 20°C

CENTRAL NERVOUS SYSTEM STIMULANTS. *Poikilotherms* Kunde (1860) observed that if frogs were injected with a strychnine solution and placed in water at 1°, 16° and 31°C , those at 31°C developed convulsions first Strychnine convulsions, produced in frogs at room temperature, disappeared upon placing the animals in water at 37°C (Foster, 1873) Blume (1930) found the rate of action of strychnine in fish (*Carassius vulgaris*) to increase with increasing water temperature

Githens (1913) made a more thorough study of the influence of temperature upon the action of strychnine in frogs. With a dose of 0 6 mgm per kgm , convulsions occurred in frogs at all temperatures from 5° to 30°C , while with a dose

of 0 3 mgm per kgm , convulsions occurred only at 5°C and above 24°C These optimum temperatures correspond to the optimum temperatures at which reflex activity of the spinal cord of frogs (*R temporaria* and *R esculenta*) is reported to occur (Storm van Leeuwen and van der Made, 1916)

Schlomovitz and Chase (1916) and Schlomovitz and Machlis (1926) studied the influence of temperature upon the time of onset of convulsions following strychnine administration to frogs (*R pipiens*) Over a temperature range of 1° to 36°C the time between injection (into the dorsal lymph sac) and the appearance of convulsions decreased progressively with increase in temperature If the heart rate was varied independently of body temperature, the time between injection and convulsions was only very slightly changed, so that the results were attributed to other causes than increase in heart rate with increase in temperature It is likely that at high temperatures an increased rate of absorption resulted from increased circulation in the region of injection

Lambruschini (1938) and Penhos (1944) found the $LD_{50}$ for strychnine sulfate in the toad (*Bufo arenarum* Hensel) to decrease with increase in temperature at 5°, 18° and 35°C the $LD_{50}$ was 120, 50 and 2 5 mgm per kgm respectively (Penhos) At 5°C the interval between injection and appearance of the convulsions with the $LD_0$ was 20 minutes, while at 35°C it was 6 minutes Führer and Breipohl (1933) reported a lower MLD for strychnine in the frog at 30°C than at 20°C

Gast (1943) found a lower fatal dose of strychnine nitrate, given to frogs (*R temporaria* and *R esculenta*) by the ventral lymph sac, at 29°C than at lower temperatures The time from injection to the appearance of convulsions was 7 hours at 2°C and 20–40 minutes at 29°C This difference, however, was reported to disappear upon intravenous injection, and convulsions then appeared immediately in both cold and warm frogs It thus seems probable that differences in rate of action which have been found after injection into the lymph sac are due to differences in absorption rates at different temperatures Gast found the time between injection and recovery from convulsions to be dependent upon the temperature For example, with 0 75 mgm per kgm , recovery occurred after 40, 16, 6 and 3 hours at 1°C , 10°, 20° and 29°C In nephrectomized frogs the same relationship between recovery-time and temperature was found as in normal frogs so that destruction of strychnine by the tissues was thought to be the most important mechanism of detoxification Penhos (1944) found a slightly greater mortality for strychnine in toads after ligation of the ureters

Strychnine oxide is not effective in producing convulsions until it is reduced to strychnine in the body In frogs the latent period between injection and appearance of convulsions with 20 mgm per kgm was found to be 2–3 days, 1½ days, 9–12 hrs and 1–2 hrs at temperatures of 1°, 10°, 20° and 29°C (Gast, 1943) This latent period was not altered by intravenous injection As with strychnine, the time for recovery following administration of strychnine oxide was longer at low temperatures than at high ones Gast and others reported that at 1–2°C the convulsions produced by strychnine differed from those at 30°C by their

longer duration, heightened tonus between convulsions and lack of "blitzartig" character

These differences probably result from the effects of cold upon the activity of the nervous system rather than from a different type of action of strychnine at the lower temperatures. Cold enhances the reflex activity of the spinal cord in mammals (Grundfest, 1941) Using single frog axons, Schoepfle and Erlanger (1941) found that cold lowered the excitability, and increased the height and duration of the spike, but the descent was prolonged more than the ascent The amplitude of spontaneous oscillations in excitability was increased by cold and by strychnine (Erlanger et al , 1941)

Other central nervous system stimulants have not received the attention that has been accorded strychnine Luchsinger (1878) reported that with picrotoxin the time from injection to appearance of convulsions in frogs was greater at lower temperatures, from 0° to 32°C The fatal dose of picrotoxin in frogs was found to be 9 mgm per kgm at 30°C and 30 mgm. per kgm at 20°C (Führer and Breipohl, 1933) The time between injection and death was about 7 times as long at 20°C as at 30°C

In the toad (*Bufo arenarum* Hensel) caffeine, injected into the ventral lymph sac, was found to be more toxic at 4°C than at 33°C (Günther and Odoriz, 1944) At 4°C , in the toad, caffeine produces a marked decrease in urine volume (in contrast to diuresis at 36°C ) and more intense hypertonia than at higher tem perature (Günther and Odoriz, 1944, 1945) The protective action of the higher temperature could not be explained on the basis of increased excretion, since only about 10 per cent of the total dose was excreted by the kidney at 36°C As the authors suggest, the lower toxicity of caffeine at high temperatures is probably due to the more rapid detoxification (demethylation) of the drug with increase in temperature

*Homeotherms* In mice, cooled to 20°C body temperature, Leser et al (1940) found that survival after 3 mgm. per kgm. strychnine sulfate was prolonged 3 5 times compared to uncooled controls. Mortality was greater in the cooled ani mals (100 per cent) than in controls (72 per cent) Gast (1943) cites unpub lished data indicating increased toxicity of strychnine in hypothermic mammals

Cats anesthetized with barbital were cooled to a body temperature of 20–23°C , and the action of analeptics injected intravenously compared with the action in anesthetized controls at 38°C (Stormont and Hook, 1941) In the hypothermic animals the lethal dose of picrotoxin was about twice that in normal animals, the toxicity of metrazol was unchanged, and strychnine was slightly more toxic than in controls.

The toxicity of metrazol in mice was not significantly different at a body tem perature of 20°C from that at 37°C (Leser et al , 1940) The time between intraperitoneal injection and appearance of convulsions was 11 times as long at 20°C as at 37°C

Coramine and lobeline were more toxic in dogs under urethane-morphine anes thesia at body temperatures of 30°C and below than at 38°C (Grosse-Brockhoff and Schoedel, 1943c) Doses which were active at normal body temperature

were often fatal in the hypothermic animals, and smaller doses were without effect The increased toxicity during hypothermia was attributed to an increased vagal action (see acetylcholine) Evidence of increased toxicity of analeptics in hypothermic animals has also been obtained by Jarisch (1943) These and other analeptic drugs were ineffective in "awakening" animals during hypothermia (Grosse-Brockhoff and Schoedel, 1943c)

Caffeine with sodium benzoate, injected subcutaneously in mice in a dose of 178 mgm per kgm , was less toxic at an environmental temperature of 30°C than at 10°C (Günther and Navarro, 1945)

CENTRAL NERVOUS SYSTEM DEPRESSANTS *Poikilotherms and isolated preparations* One of the experimental findings which forms the basis of the Meyer-Overton theory of narcosis is the parallel change in partition coefficient and narcotic action with change in temperature reported by H H Meyer in 1901 Meyer found, using tadpoles, that a rise in temperature (from 3° to 30°C ) increases the oil/water partition coefficient and the narcotic action of chloral hydrate, ethyl alcohol and acetone, on the other hand, a rise in temperature decreases both the partition coefficient and narcotic action of salicylamide, benzamide and monoacetin Similar experiments have been performed by others Although some of the results are conflicting, the weight of the evidence confirms and strengthens Meyer's results Henderson (1930) has reviewed this subject and cited the more pertinent papers, references to the influence of temperature upon alteration of surface tension by narcotics are also given Recently McElroy (1943) has reported the effect of temperature upon narcotic action (in the case of luminous bacteria) and has discussed the relationship of lipoid solubility and enzyme denaturation to narcosis

The rate of action of ethyl alcohol and chloroform as a function of temperature was investigated by Veley and Waller (1910) on isolated frog muscle The data were found to yield straight lines when the logarithm of the decrement of time for complete inhibition of contraction was plotted as a function of the logarithm of the increment of absolute temperature (cf Bělehrádek, 1935) The $Q_{10}$ for the inhibition of muscle contraction was 2 04 for alcohol, 1 63 for chloroform and 2 0 for ether (Waller, 1909)

The temperature characteristic ($\mu$) for oxygen consumption of the newt under chloretone anesthesia was the same as in unanesthetized animals, it was altered under pentobarbital anesthesia (Gordon and Pomerat, 1942)

Toads at 3°C tolerate doses of morphine sulfate which are fatal at 20° or 30°C (Lambruschini, 1938) In general the fatal dose was found to decrease with increase in temperature

The effect of temperature upon the toxicity of some other hypnotics in frogs was reported by Richards (1941) The $LD_{50}$ for paraldehyde was 4 4 grams per kgm at 10°C , 3 9 gms per kgm at 20°C and 2 5 gms per kgm at 30°C The $LD_{50}$ for pentothal and pentobarbital decreased with increase in temperature at 20°, 30° and 35°C However, for these two drugs, the $LD_{50}$ was lower at 10° than at 20°C

*Homeotherms* Cameron (1939) reported that rats anesthetized with a given

dose of pentobarbital died if placed in a draft during November, but invariably recovered if kept warm Similar observations had been made earlier L. Hermann (1867) found that rabbits tolerated alcohol better if they were warmed than if they were allowed to cool spontaneously at room temperature Brunton (1874, 1885) states that death could be prevented in animals poisoned with chloral by warming In the above experiments the animals which were not warmed unquestionably suffered considerable fall in body temperature, since anesthetics suppress reflex responses to cold (Hemingway, 1940)

The newborn of most species of mammals (with the outstanding exception of the guinea pig) are more resistant to anoxia than the adults of the species (cf Windle, 1943) This is true whether the animals breathe nitrogen or other inert gases, or whether gaseous anesthetics such as nitrous oxide or cyclopropane are used (Fazekas, Alexander and Himwich, 1941) These authors demonstrated that elevation of environmental temperature shortens the survival time, so that the poikilothermia of the infant is one of the factors concerned in the better survival Barrows (1933) found that the survival time of mice in ether vapor decreased with increasing age of the animals, and called attention to the relationship between survival and the development of temperature regulation in mice as demonstrated by Sumner (1913) More recently Barrows and Dodds (1942) determined the survival time of mice of different ages in ether or chloroform vapor at environmental temperatures of 22°, 28°, 35° and 40°C For a given age up to about three weeks the survival time increases with a decrease in environmental temperature, and, since mice of this age are essentially poikilotherms, survival time increases with a decrease in body temperature as well

In rats 20 mgm per kgm. morphine sulfate was fatal in some animals exposed to an environmental temperature of 3°C , but not in animals at room temperature (Herrmann, 1941) Fall in body temperature to 23°C in 6 hours at 3°C occurred so that it is probable that hypothermia was a contributing factor in the mortality Similar results were obtained with paraldehyde and pentobarbital

In mice cooled to 20°C , Leser et al (1940) found the survival time with 350 mgm. per kgm. morphine sulfate to be increased in the cooled animals and the toxicity slightly, but probably not significantly, increased.

Raventos (1938) carried out the most complete study of the effect of environmental temperature yet reported on the action of barbiturates The duration of anesthesia produced in mice by intraperitoneal administration of 100 mgm per kgm sodium evipal was 2 5 times as long at 20° as at 30°C and was shorter at 40° than at 30°C The $HD_{50}$ (median hypnotic dose) for the same drug was 39 mgm per kgm at 30° and 45 mgm per kgm. at 20°C , the $LD_{50}$ was the same at both temperatures. With sodium phenobarbital the $HD_{50}$ was 105 mgm per kgm at 30° and 90 mgm per kgm at 20°C , the $LD_{50}$ decreased from 234 mgm per kgm. at 30° to 162 mgm per kgm at 20°C The difference in the effect of temperature upon the lethal doses of the two barbiturates was accounted for on the basis of a difference in time required to produce death With evipal death occurred soon after injection, before body temperature of the mice was reduced, with phenobarbital death occurred 3 to 24 hours after injection at a time when

the body temperature of the animals reached that of the environment, so that hypothermia was a contributory cause of death in the case of animals given phenobarbital and exposed to 20°C Raventos emphasizes that part of the discrepancies which have been previously reported in both lethal and hypnotic doses of barbiturates are due to failure to control environmental temperature

Gaylord and Hodge (1944) reported that in both normal and castrate female rats given 30 mgm per kgm pentobarbital subcutaneously at 13°, 23° and 37°C, the duration of sleep increased with decrease in environmental temperature

The reports of Cameron (1939) and Gaylord and Hodge (1944) using pentobarbital, and of Raventos (1938) using evipal, indicate that rats and mice sleep longer with a given dose of the barbiturate at lower environmental temperatures Since, in anesthetized animals (particularly in mice), the body temperature falls rapidly toward that of the environment, this difference in sleeping time may be due to a decreased rate of detoxification of these barbiturates at the lower temperatures Since it is known that all common barbiturates, with the exception of sodium barbital, are largely detoxified in the body (Masson and Beland, 1945), it would be desirable to compare the sleeping time of mice at different temperatures using barbital and one of the rapid-acting group such as pentobarbital This was done in this laboratory (Fuhrman, 1946) Forty six white mice weighing 14–30 grams were injected intraperitoneally with 37 5 mgm per kgm sodium pentobarbital or 290 mgm per kgm sodium barbital They were maintained at room temperature (25°C ) until anesthesia developed, then half of each group were cooled to a body temperature of 27°C , and half were warmed to a body temperature of 37°C With sodium pentobarbital the mean sleeping time at 37°C was 17 5 minutes, while at 27°C it was 61 8 minutes The difference between the means was significant ($P < 0\ 0001$) With sodium barbital the mean sleeping time at 27°C was 331 5 minutes, while at 37°C it was 314 5 minutes The difference between these means was not significant ($P < 0\ 2$)

These data are in agreement with the hypothesis that for a drug which is chemically detoxified by the tissues (pentobarbital) the action is prolonged by hypothermia, while for a drug which is not detoxified (barbital) the duration of action may not be influenced by low body temperatures

Ethyl alcohol metabolism The rate of alcohol metabolism in the frog (*R temporaria*) increases with increase in temperature, yielding a $Q_{10}$ of 1 97 over the range 1°C to 29°C (Nicloux, 1931) In 24 hours 14 7 per cent of the alcohol administered was metabolized at 1°C , while 97 per cent was destroyed at 29°C These data are consistent with the view that alcohol is oxidized enzymatically (chiefly in the liver) and that reduction in the temperature at which the oxidation occurs results in a slowing of the rate of oxidation

In homeotherms the rate of metabolism of alcohol is higher in small animals with high metabolic rates than in larger animals (LeBreton, Nicloux and Shaeffer, 1935, Newman and Lehman, 1937) In a given species, however, attempts to increase the rate of alcohol metabolism by increasing the metabolic rate with thyroxin (Widmark, 1935, Harger and Hulpieu, 1935), dinitrophenol (Newman and Tainter, 1936) or by exposure to a cold environment (LeBreton, 1934) did

not succeed in significantly altering the rate of combustion of alcohol By elevation of body temperature by means of diathermy, slight increases in rate of metabolism of alcohol have been reported (Fleming and Reynolds, 1935, Ewing, 1940) The increase in temperature obtained was so small that changes in rate of alcohol metabolism are difficult to demonstrate Fuhrman and Fuhrman (unpublished) determined the rate of metabolism of alcohol in rabbits at body temperatures of 38°, 30°, 27.5° and 25°C after intravenous injection of 0 78 grams per kgm. alcohol The rate of combustion of alcohol in all of the hypothermic animals was lower than that at normal body temperatures, the rate of combustion was 100 mgm per kgm per hour at 25°C compared to 156 mgm. per kgm. per hour at 38°C These data, taken together with those demonstrating failure to increase the rate of combustion of alcohol by an increase in metabolic rate, show that in homeotherms, as well as in poikilotherms, the rate of alcohol metabolism is dependent upon body temperature

Dinitrophenol. The calorigenic effect of 2–4 dinitrophenol has been reported to be less apparent in homeotherms at low environmental temperature than at a temperature in the neighborhood of thermal neutrality (Magne, Mayer and Planteſol, 1932, Ghaja and Dmitrijevic, 1933, Tainter, 1934) At low environmental temperatures animals injected with large doses of dinitrophenol may become hypothermic Tainter (1934) found that the body temperature of rats given 30 mgm. per kgm. dinitrophenol fell 4 1°C in 4 hrs. at 2°C air temperature, compared to 1 6°C fall in controls G J Fuhrman et al (1943), using mice at 6°C found that 50 mgm. per kgm resulted in a fall in body temperature to 21–22°C and death of the animals within one hour

G J Fuhrman et al. also reported the $LD_{50}$ of 2–4 dinitrophenol administered subcutaneously in mice at different environmental temperatures to be 35 7 mgm per kgm. at 6°C , 30 9 mgm. per kgm. at 25°C , and much lower yet at 40°C Body temperatures of the mice fell at 6°C and 25°C

In order to elucidate the influence of cold upon the calorigenic effect of dinitrophenol, Hall et al (1937) investigated its effect upon oxygen consumption and shivering in anesthetized cats rendered hypothermic by immersion in cold water In the hypothermic cats dinitrophenol greatly decreased or abolished shivering in spite of the continuation of conditions normally evoking shivering Thus dinitrophenol depresses the mechanism for chemical defense against cold When allowance was made for the oxygen cost of shivering it was shown that the calorigenic action of dinitrophenol on the cat was augmented by hypothermia, and that the intensity of the calorigenic effect was greatest over the body temperature range 33° to 35°C Subsequent work of Hall and Chamberlin (1937) demonstrated a synergic calorigenic action of dinitrophenol and epinephrine when the latter was infused intravenously at a physiological rate Epinephrine secretion during hypothermia was suggested as a major factor in enhancement of dinitrophenol action in hypothermic animals.

Hall et al. (1937) suggested that the depression of shivering by dinitrophenol was of central origin and that "should dinitrophenol increase the local cellular metabolism of the portion of the gray matter constituting the heat regulatory

center, to a sufficient degree, its threshold of excitation by cooled blood or by receptor impulses would be increased It would result that the center woul thermostatically adjusted to regulate at a lower body temperature than norm Evidence for such a hypothesis was provided by Fuhrman and Field (1942) found that the maximum stimulation of oxygen consumption of excised rat *bral cortex by dinitrophenol occurred at 32°C* Thus in excised rat brain an the intact cat the maximum stimulation of oxygen consumption by dinitroph occurs at approximately the same temperature In other excised tissues temperature at which maximum stimulation occurs is different, 22°C for kidney cortex (Fuhrman and Field, 1942) and 35°C for rat skeletal mu (Hollinger, 1944–45)

HORMONES *Epinephrine* Langlois (1897) observed that the increas heart rate of the turtle, produced by epinephrine, persisted longer at 15°C at 37°C He then found that the response to epinephrine injection in mam was prolonged in an animal cooled to 35°C (Langlois, 1898) A similar pro gation was noted by Lewandowsky (1899) for the dilatation of the cat's p Elliott (1905) sought an answer to the question of whether this prolongation due to a delayed disappearance of epinephrine or to alteration of the muscle result of the reduced temperature He concluded, from experiments with local and general cooling, that cooling increases the duration of contraction muscle, but that the contraction produced by epinephrine administration is longed beyond that of the cooled controls McGuigan (1938) reported increase in blood pressure of dogs in response to epinephrine to be less at peratures above normal, but did not attribute the change in response to a r rapid destruction of epinephrine at the higher temperatures

The effect of temperature upon the response of the nictitating membran the cat to epinephrine was investigated by Fuhrman et al (1944) They fo that, for a given dose of epinephrine, the response of the nictitating memb became progressively greater as the body temperature was reduced Over temperature range 12 9°C to 42 3°C the logarithm of the duration of cont tion of the nictitating membrane was a linear function of the temperat Regional warming of the liver of hypothermic cats (by means of long wave thermy) without change in either rectal temperature or temperature of the n tating membrane, shortened the response of the membrane to a given dos epinephrine At reduced levels of body temperature ventricular fibrillation produced by doses of epinephrine which produced no evidence of cardiac irr larity at higher temperatures These results were interpreted to mean that enzymatic detoxification of epinephrine is slower at low temperatures, a gi dose therefore, produces a longer and more intense action during hypother than at normal body temperature In agreement with this view the fatal dos epinephrine in frogs is greater at 20°C than at 30°C (Führer and Breip 1933)

In contrast to these findings on intact animals, a number of experiments *isolated heart and blood vessels have shown that the maximum activity* epinephrine is exhibited at about 37°C On the isolated rabbit heart the actr

of epinephrine was reported to be greater at 37° than at 18°C (Panella, 1907) The amplitude of contraction of the perfused frog heart was increased more at temperatures above 18°C than below (Sprinceana, 1915) The rate response of the perfused frog heart to epinephrine increased with increase in temperature (Barlow and Sollmann, 1926) The optimum temperature for action of epineph rine upon perfused intestinal blood vessels was 37–39°C (Kosuge, 1935) All of these experiments differ from those reported above in that no mechanism exists for epinephrine detoxification, since the liver is not a part of the perfusion system. In the intact animal, then, the rate of destruction of epinephrine appears to be the principal factor determining its intensity of action

*Insulin Poikilotherms* Krogh and Rehberg (cf Macleod, 1926, Hemmingsen, 1925) observed that frogs injected with insulin developed convulsions only after 4 to 5 days Further investigation of the rate of action of insulin in poikilotherms has shown that the length of the latent period increases with decrease in temperature

Frogs given 0 45 to 3 units of insulin developed convulsions at 30°C within 14 hours and all died, at lower temperatures death did not result, and convulsions developed at 25°C in 24–27 hours at 20°C in 43–49 hours, at 15°C in 60–70 hours and at 7°C in 120–144 hours (Huxley and Fulton, 1924) Extrapolation to 37°C indicated that frogs should develop convulsions in 1½ to 2½ hours at that temperature, this time is not greatly different from that found in rabbits Olm sted (1924) confirmed these observations in frogs and later found in toads that convulsions occurred 18, 24 and 36 hours after insulin injection at 30°, 25° and 18°C respectively (Olmsted, 1926) In the frog, in which blood sugar was elevated by exposure to high temperature (Olmsted, 1924), and in the sculpin (*Myoxocephalus*), in which the normal blood sugar is low (McCormick and Macleod, 1925), insulin administration resulted in little change in blood sugar In other poikilotherms, including frogs, turtles, snakes, fish and the Cayman, insulin produced hypoglycemia after several hours (Sordelli, Houssay and Mazzocco, 1923, Houssay and Rietti, 1924, Mann, Bollmann and Magath, 1924, Hemmingsen, 1925, Barlow, Vigor and Peck, 1931) In the brown trout (*Salmo fario*) at 4°C , and the menhaden (*Brevoortia tyrannus*) and scup (*Stenotomus Chrysops*) at 19 5°C , insulin produced a marked fall in blood sugar, and convul sions developed approximately 24–60 hours, 23 hours and 7 hours respectively, after injection In the puffer (*Spheroides maculatus*) at 19 5°C no convulsions were observed (Gray, 1928)

Gray suggests that metabolic rate may be more important than temperature in determining the time required for the production of convulsions by insulin in fish, since the trout at 4°C (convulsions in 24–60 hrs ) may have as high a metabolic rate as the catfish at 21°C (convulsions in 51–53 hrs.) Huxley and Fulton suggested that the action of insulin is not itself altered by change in temperature, but that "its speed of action is dependent upon the metabolic rate of the animal itself " Since, for poikilotherms, the metabolic rate increases with increase in temperature (Krogh, 1941), it is difficult in this case to differen tiate the effects of metabolic rate and of temperature upon insulin action In

view of the effect of temperature upon the rates of biological processes, it is quite possible that the action of insulin itself as well as the rate of metabolism is directly influenced in a similar manner by temperature

*Homeotherms* The action of insulin in mammals is influenced by environmental temperature Temperatures below about 30°C increase the metabolic rate of homeotherms Mortality is higher and convulsions appear more rapidly in rats and mice after administration of insulin if the air temperature is maintained around 30°C than if it is lower (Voegtlin and Dunn, 1923, Hemmingsen and Krogh, 1926, Hemmingsen, 1939) The $CD_{50}$ in mice is about 80 times as great at an environmental temperature of 20°C as at 40°C (Chen et al, 1943b) Rabbits, fasted for 18 hours at 33°C, injected with insulin and exposed for 7 hours at the same temperature, developed convulsions (Johlin, 1944) With the same dose of insulin and both exposure temperatures at 20°C, convulsions again appeared However, when the fasting period was at 33°C and the experimental period at 20°C, convulsions did not appear Blood sugar level was the same in all animals The author suggests that hyperventilation at 33°C produced a temporary uncompensated acidosis at 20°C which restrained the convulsions An inhibiting effect of high atmospheric $pCO_2$ on insulin convulsions has been reported (McQuarrie et al, 1940)

The action of insulin on homeotherms has also been investigated during hypothermia Cassidy, Dworkin and Finney (1925a) found that in dogs and cats (fasted 24 hrs and anesthetized with amytal) insulin lowered the blood sugar with approximately the same rapidity at body temperatures of 38° and 25°C In some animals at 25°C, however, there was a delay of 40–60 minutes before the fall in blood sugar commenced, but after that time the form of the blood sugar curve did not differ appreciably from that at 38°C

Krogh (cf Macleod, 1924) observed that at room temperature insulin rarely produced convulsions in mice, but only a comatose condition and a fall in body temperature, but that if the animals were kept at an environmental temperature of 28°C (and a fall in body temperature prevented), convulsions of the usual type were seen In dogs and cats with blood sugar below 45 mgm per cent, insulin convulsions did not occur at rectal temperatures of 25°C, even though in two dogs blood sugars as low as 16 and 36 mgm per cent were found Convulsions did occur when the animals were re-warmed to 38°C (Cassidy, Dworkin and Finney, 1925-b) Lowering of the blood sugar by means of insulin abolished shivering in hypothermic cats and dogs, shivering could be restored by means of glucose (Cassidy et al, 1925-b) Fowls in which the blood sugar was reduced to about 40 mgm per cent by means of insulin did not at once shiver upon immersion in water at 20°C (which produces shivering in the normal hen), but shivering began coincidentally with a rise in blood sugar (Cassidy, Dworkin and Finney, 1926) Woodchucks (*Arctomys monax*), given an amount of insulin sufficient to produce marked hypoglycemia, lose the power of temperature regulation, and hypothermia occurs when they are placed in a cold environment (Dworkin and Finney, 1927)

Tyler (1939) found that the body temperature of rabbits could be reduced to

about 28°C in 3 hours by the administration of 3 U /kgm. insulin, warming the animals with alcohol, and placing them in front of a fan If animals were first cooled to 25–28°C , then given 0 5 U /kgm. insulin I V., the rate of fall of blood sugar was about the same as in animals at normal body temperature, but 6–10 hours were required for it to return to normal in the cooled animals as compared to 3–5 hours in uncooled controls This prolonged hypoglycemia may be due to decreased glyconeogenesis at low temperature or to a prolongation of insulin action Survival after massive doses of insulin was prolonged at body temperatures of 25°–30°C The hypothesis was offered that both the increase in the survival period and the inhibition of convulsions in cooled animals receiving insulin are the result of the decreased metabolic rate and the subsequent decreased demand for substrates by the vital centers of the brain (Tyler, 1939) Brain damage occurred earlier in cats rendered hypoglycemic by insulin when the body temperature was high than when very marked falls occurred This was investigated more completely in cats which were maintained at body temperatures of 31°–34°C or 36°–39°C (Tyler, 1940) The extent of brain damage (determined histologically) was greater in those at 36°–39°C for a given duration of hypoglycemia than in those at 31°–34°C When the body temperature was maintained at normal levels it was considered that a greater relative degree of tissue anoxia resulted than when the temperature was permitted to fall.

Since the mechanism of production of insulin convulsions is not definitely known (cf Jensen, 1938, p 157), the reason for prevention of convulsions by hypothermia is not clear It seems probable that convulsions occur at blood glucose concentrations of essentially zero (Dotti and Hrubetz, 1936) and that they thus depend upon failure of carbohydrate supply to the brain (Gerard, 1938) At low temperatures, when brain metabolism is greatly reduced, convulsions may be prevented by substrate concentrations inadequate to prevent them at normal body temperatures

*Thyroid* Unfortunately the effect of thyroxin or thyroid administration has not been thoroughly studied in homeotherms at body temperatures other than normal. O'Connor (1936) reported that thyroid increased the oxygen consumption of anesthetized, curarized rabbits about 50 per cent at all body temperatures from 22° to 39°C Other workers have reported chiefly the results of experiments at varied environmental temperature, which has a marked effect upon the survival time of mammals treated with thyroxin or desiccated thyroid. Nolan and Kinney (1919) reported that rats given 0.2 gram of thyroid daily survived for 7 days at an environmental temperature of 32°C , for 22 days at 25°C and for 35 days at 18°C At 33°C environmental temperature rabbits survived when given 50–75 mgm per kgm desiccated thyroid daily, while a similar body weight is maintained (Draize and Tatum, 1932) More complete data of a similar nature are given by Bodansky, Pilcher and Duff (1920), who found that the period of survival of rats given 2 mgm thyroxin daily increased as the environmental temperature Dempsey and Astwood (1943) found that rats treated with thiouracil, that daily doses of 9 5 micrograms per 100 gms body weight and 1 7 micrograms of thyroxin daily were required to maintain the same

roid gland at environmental temperatures of 1°, 25° and 35°C respectively Guinea pigs, given 0 3 mgm thyroxin daily, showed a greater increase in rectal temperature than did untreated animals when placed at a high environmental temperature At the high temperature $CO_2$ production increased in the thyroxin-treated animals and decreased in normal animals (Okamura, 1939)

In contrast to the marked effects of thyroid or thyroxin in increasing the metabolic rate of homeotherms, the metabolic rate of frogs and small fishes does not appear to be increased by the administration of either substance (Drexler and v Issekutz, 1935, Henschel and Steuber, 1931) Tadpoles of *R temporaria* exposed to thyroid in solution and then placed at environmental temperatures of from 3° to 30°C undergo complete metamorphosis only at temperatures above 5°C (Huxley, 1929) Below 5°C metamorphosis is not complete, even when the animals are later removed to room temperature The implications of this finding are discussed by Needham (1942)

MISCELLANEOUS DRUGS *Acetylcholine* Clark (1926) reported that temperature produced no certain effect upon the amount of action of acetylcholine on isolated frog heart or rectus abdominis An increase in temperature, however, increased both the rate of action and the rate of washing out Laubender and Kolb (1936) obtained similar results on the isolated frog rectus abdominis

The slowing of the heart produced by intravenous administration of acetylcholine in dogs was much greater at 25°C body temperature than at 36°C (Tournade et al , 1938, Grosse-Brockhoff and Schoedel, 1943c) Arrhythmias frequently occurred at the lower temperature with doses which did not disturb the cardiac rhythm at 36°C Vagal stimulation, at an intensity ineffective at normal temperature, produced marked effects at 25°C These changes were suggested as being due to a decreased activity of choline esterase at low temperature Acetylcholine, whether injected or formed as the result of vagal stimulation, would thus be present in higher concentration and for a longer period of time at 25°C than at 36°C

*Dicumarol* The influence of fever upon the action of dicumarol, as measured by prothrombin time, has been studied by Richards (1943) Prothrombin time, determined 24 hours after the administration of dicumarol, was five times as long in rats with a mean body temperature of 39 6°C as in rats with a mean body temperature of 37 3°C These data are in accord with the view that reduction in plasma prothrombin level is dependent upon a chemical or enzymatic reaction involving dicumarol

*Carbon dioxide* Frogs were not anesthetized with 25 per cent $CO_2$ during several days' exposure, and were anesthetized only after several hours with 50 per cent $CO_2$ (Winterstein, 1919) Narcosis in rats and dogs was produced by 11 per cent $CO_2$ at an environmental temperature of 5°C in 3 to 6 hours (Barbour and Seevers, 1943) At room temperature such $CO_2$ concentration does not result in narcosis Body temperature of the narcotized animals fell and it was necessary to raise the environmental temperature after induction of narcosis in order to prevent death from hypothermia Barbour and Seevers suggest that a sudden increase in tissue $CO_2$ tension decreases total oxidative metabolism and reduces

activity of the tissues, including the central nervous system At 5°C decrease in total heat production results in lowering of body temperature to the point of narcosis

*Oxygen* The effects of oxygen at increased pressure have recently been reviewed by Bean (1945) Poikilotherms appear to be more resistant to increased oxygen pressure than are homeotherms. The toxic action of oxygen at high pressure is enhanced by increase in environmental temperature (References and details are given by Bean )

*Tetanus toxin* This toxin is reported to be relatively inactive in frogs at room temperatue and below, but to become toxic upon warming the animals (*Courmont and Doyon*, 1893)

*Abrin* Abrin was reported by Lesné and Dreyfus (1908) to be several hundred times more toxic in frogs at 29°C than at room temperature Guinea pigs maintained at an environmental temperature of 39°C were killed by a dose which was not fatal in animals at lower temperatures.

*Atoxyl* Atoxyl (sodium arsanilate) was found to be much less toxic in frogs than in mammals (Muto, 1910) Sanno (1911) determined the lethal dose in frogs at 8°C to be about 15 grams per kgm and at 37°C to be about 1 3 grams per kgm These results may be explained by the conversion of sodium arsanilate by the tissues to a more toxic reduction product, a reaction which is more rapid at higher temperatures.

*Nicotine* Hill (1909) determined the $Q_{10}$ of the action of nicotine on isolated frog muscle to be 2 8 between 7° and 17°C Laubender and Kolb (1936) obtained a $Q_{10}$ of 2.2 This, together with concentration action data, was taken to indicate a chemical mode of action, but Clark (1937) points out that there is no guarantee "that at different temperatures fixation of equal quantities of drug will produce an equal effect "

In mice cooled to a body temperature of 20°C , nicotine is more toxic than in control animals at normal body temperature (Leser et al , 1940)

*Procaine and cocaine* Procaine, injected intramuscularly, was found to be most toxic in mice during the summer months (Sievers and McIntyre, 1937) The effects of environmental temperature were investigated more completely by determining the fatal dose in mice exposed to a given temperature ranging from about −10°C to 40°C for two hours before injection The largest minimum lethal doses were found in the temperatue range 7 to 24°C , with decrease in MLD at temperatures both below and above this range Below 7°C the body temperature of the mice was probably reduced by the preliminary exposure period

In mice cooled to 20°C body temperature, both procaine and cocaine were more toxic than in control animals and the survival time was prolonged in the cooled animals (Leser et al , 1940)

Discussion The complexity of the effects of temperature upon the action of drugs is at once apparent from the foregoing consideration It is however, possible and highly desirable to classify the available data in such a way that general conclusions may be drawn It is no longer possible to accept without

reservation the conclusion of Richet (1889, p 214) "les actions toxiques, étant des actions chimiques, varient avec le température de l'animal Elles sont d'autant plus actives que la température de l'animal est plus élevée" As Bělehrádek (1935, p 90) clearly points out "In the action of various chemicals upon living cells and organisms under varied temperature, two separate phenom ena must be clearly distinguished, viz , (a) the effect of temperature upon the rate at which a given substance permeates into the system or at which it produces definite changes in the system, and (b) the variations of the effective concentra tion, or of the final concentration, or of the mode of action, of such a substance, with temperature "

*Rate of action* When pharmacologically active agents are administered to intact animals at different levels of body temperature, the rate at which they act is usually measured by the elapsed time between administration and the oc currence of a response This latent period is determined (1) by the time required for the drug to reach the site of action in sufficient concentration to produce an effect, and (2) by the time required for the interaction between the drug and the tissues

The time between administration and the appearance of effect is very greatly influenced by the route of administration Following intravenous administra- tion, the effect of temperature upon this phase of action is a result of the effect of temperature upon the circulation time In man, during general hypothermia, the circulation time is found to be two to three times as long as at normal body temperature (Oppenheimer and McCravey, 1941) Following subcutaneous or intramuscular administration, it is to be expected that rate of absorption will be dependent upon blood flow in the region of injection Local cooling, operating through local reduction of blood flow, is an effective means of delaying absorption Part of the effect of low temperature in decreasing the toxicity of digitalis in frogs, when the drug is administered via the lymph sac, is the result of the slower absorption of digitalis at low temperature (Sollmann et al , 1915)

The effect of temperature upon the rate of interaction between the drug and the tissues of the animal is often obscured by the effect of temperature upon the rate of absorption This interaction is believed to be chemical in many in stances It is therefore to be expected that the reaction will be temperature- sensitive, and that the rate at a given temperature will be in general about two to three times the rate at a temperature 10°C lower ($Q_{10} = 2$–$3$) Experimental data describing the effect of temperature upon the rate of interaction of drugs and tissues have provided temperature coefficients of about this magnitude (Clark 1937, Bělehrádek, 1935) Although such temperature coefficients indicate that the actions produced are chemical in nature, it must be remembered that changes in temperature produce alterations in the tissues themselves changes in rate of nerve conduction, metabolic rate, viscosity, etc (cf Clark, 1937, Lucas, 1908, Samojloff, 1925) There may still be doubt, in spite of such temperature coefficients, as to whether the process is physical or chemical

*Intensity and duration of action* Intensity of drug action is dependent upon the concentration of the drug obtaining at the site of action, and upon physiolog-

ical factors which may alter the response of the tissue acted upon The concentration of drug attained in a tissue is the resultant of the processes of absorption, excretion and detoxification All of these processes may be influenced by temperature, and each may be altered to a different degree The rate at which an inactive form of drug is changed into an active form may also be altered by temperature

The duration of action of a drug is determined by the length of time during which a concentration above the threshold level is maintained at the site of action This is influenced both by the rate of absorption or of activation and the rate of excretion or destruction. The influence of temperature upon the rate of absorption has already been mentioned. Change in body temperature also has a profound effect upon the rate at which a drug is destroyed by the tissues Although these detoxification processes are in most cases not yet well enough understood to describe complete systems, it is clear that for a number of drugs (acetanilide, histamine, atropine, epinephrine, alcohol) these reactions are enzymatic in nature (Bernheim, 1942) The short-acting barbiturates are probably also detoxified enzymatically Thus it must be true, in view of the very well established effect of temperature upon rate of enzyme action (Sizer, 1943), that decrease in body temperature will slow the enzymatic detoxification of these substances, the $Q_{10}$ for such a process should lie in the range 2 to 4 Experimental evidence that the duration of action of acetylcholine, caffeine, epinephrine, alcohol and pentobarbital are markedly increased at low body temperature levels has been provided In contrast, the action of barbital (which is largely excreted in the urine) is not prolonged during hypothermia It is probable that other examples of this type of temperature effect will be found

Colchicine and atoxyl are changed by the tissues to more active products and thus, by reason of slower activation, are less toxic at low body temperatures

The effect of body temperature upon the rate of excretion of drugs by the kidney does not appear to have been investigated It is reported that kidney function is maintained during hypothermia in man (Smith, 1942), so that large alterations in urinary excretion by changes in body temperature may not be expected to occur

Changes in lethal dose with changes in temperature may result from a greater effect of temperature upon either rate of absorption or rate of elimination Variations in lethal dose produced by changes in body temperature are complicated at both extremes by the effect of temperature upon the organism For example, during hypothermia the summated depressant effects of cold and of anesthetics may lead to respiratory failure with lower doses of anesthetic than are lethal at normal body temperature

When the contributions of experimental investigation of the effect of temperature upon drug action carried out during the last 60 years are considered, there is even more reason today than there was in 1885 to agree with Brunton that "All these things show that the *definition* of the *action of a drug* must be still further modified, and we must define it as the reaction between the drug and the various parts of the body at a *certain temperature*"

I am grateful to Dr B Günther for permitting me to cite unpublished observations and to Geraldine J Fuhrman for much assistance in the preparation of the manuscript

## REFERENCES

ADOLPH, E F Biol Bull **62** 112, 1932
ARIEL, I , F W BISHOP AND S L WARREN Cancer Res **3** 448, 1943
BAKER, W F Lilly Scientific Bull Ser 1, no 3, p 71, 1913
BARBOUR, J H AND M H SEEVERS J Pharmacol and Exper Therap **78** 296, 1943
BARLOW, O W AND T B SOLLMANN J Pharmacol and Exper Therap **28** 325, 1926
BARLOW, O. W , W M VIGOR AND R I PECK J Pharmacol and Exper Therap **41** 229, 1931
BARROWS, E F Proc Soc Exper Biol and Med **30** 1290, 1933
BARROWS, E F AND H DODDS Am J Physiol **137** 259, 1942
BAZETT, H C J A M A **111** 1841, 1938
BEAN, J W Physiol Revs **25** 1, 1945
BĚLEHRÁDEK, J Arch internat de physiol **35** 1, 1932 Temperature and living matter Borntraeger, Berlin, 1935
BENEDICT, F G Carnegie Inst of Washington, Publ no 425, 1932
BENEDICT, F G AND R C LEE Carnegie Inst of Washington, Publ no 497, 1938
BERNHEIM, F The interaction of drugs and cell catalysts Burgess, Minneapolis, 1942
BILLINGER, O Wien klin Rundschau **10** 769, 1896
BLAIR, A P , C C HARGREAVES AND K K CHEN Proc Soc Exper Biol and Med **45** 209, 1940
BLANCHARD, R Compt rend Soc de biol **55** 734, 1120, 1903
BLUME, W Arch exper Path und Pharmakol **149** 186, 1930
BODANSKY, M , J F PILCHER AND V B DUFF J Exper Med **63** 523, 1936
BRACHET, J AND F BREMER Arch internat de physiol **51** 195, 1941
BRITTON, S W Am J Physiol **67** 411, 1924
BRUNTON, T L J Anat and Physiol **8** 332, 1874 A textbook of pharmacology, therapeutics and materia medica Lea Brothers, Philadelphia, 1885
CAMERON, A T AND T J BROWNLEE Quart J Exper Physiol **7** 114, 1914
CAMERON, G R Proc Roy Soc Med (London) **32** 309, 1939
CASSIDY, G J , S DWORKIN AND W H FINNEY Am J Physiol **73** 413, 1925a Am J Physiol **73** 417, 1925b Am J Physiol **75** 609, 1926
CHEN, K K AND A L CHEN J Pharmacol and Exper Therap **47** 295, 1932
CHEN, K K AND C L ROSE Proc Soc Exper Biol and Med **49** 351, 1942
CHEN, K K , R C ANDERSON, F C HENDERSON AND C A MILLS Proc Soc Exper Biol and Med **53** 200, 1943a
CHEN, K K , R C ANDERSON, F A STELDT AND C A MILLS J Pharmacol and Exper Therap **79** 127, 1943b
CLARK, A J J Physiol **61** 530, 1926 General Pharmacology in Handbuch d exper Pharmakol (Heffter) Ergänzungswerk, Bd 4, 1937
COURMONT, J AND A DOYON Compt rend Soc de biol **36** 92, 1893
CRISMON, J M Arch Int Med **74** 235, 1944
CRISMON, J M AND F A FUHRMAN In preparation, 1946
CROZIER, W J J Gen Physiol **7** 201, 1924
DEFANDORF, J H J Am Pharm A **22** 599, 1933
DEMPSEY, E W AND E B ASTWOOD Endocrinology **32** 509, 1943
DILL, D B AND W H FORBES Am J Physiol **132** 685, 1941
DOTTI, L B AND M C HRUBETZ J Biol Chem **113** 141, 1936
DRAIZE, J H AND A L TATUM Arch internat de pharmacodyn et therap **43** 237, 1932
DREXLER, E AND B v ISSEKUTZ Arch exper Path und Pharmakol **177** 435, 1935

Dworkin, S and W H Finney Am J Physiol 80 75 1927
Edmunds, C W and W Hale U S Hyg Lab Bull no 48 1908
Elliott T R J Physiol 32 401 1905
Erlanger, J E A Blair and G M Schoepfle Am J Physiol 134: 705 1941
Ewing P L. Quart J Stud Alcohol 1: 483, 1940
Eyring H and J L Magee J Cell Comp Physiol 20 169 1942
Fay T and G W Smith Arch Neurol and Psychiat 45 215 1941
Fazekas J F F A D Alexander and H E Himwich Am J Physiol 134 281, 1941
Feitelberg S and E P Pick. Proc Soc Exper Biol and Med 49 657 1942
Field J F A Fuhrman and A. W Martin J Neurophysiol 7 117 1944
Fischer, H Arch exper Path und Pharmakol 135 39 1928
Fleming R and D Reynolds J Pharmacol and Exper Therap 54 236, 1935
Focke C Arch d Pharm. 245 646 1907
Foster M. J Anat and Physiol 8 45 1873
Führer H. Arch exper Path und Pharmakol 63 357 1910 Arch d Pharm. 269: 176, 1931 Arch exper Path und Pharmakol 166: 437 1932
Führer H and W Breipohl. Arch exper Path und Pharmakol 173: 146 1933
Fuhrman F A In preparation 1946
Fuhrman F A and J Field J Pharmacol and Exper Therap 75 58 1942 Am J Physiol 139 103 1943 Arch Biochem 6 337 1945
Fuhrman F A. and G J Fuhrman In preparation 1946
Fuhrman F A, J M Crismon G J Fuhrman and J Field J Pharmacol and Exper Therap 80 323 1944
Fuhrman, G J The effect of cold and dinitrophenol on the metabolism and temperature regulation of albino mice Thesis Stanford University 1943
Fuhrman G J J Field and F W Weymouth J Pharmacol and Exper Therap 79 176 1943
Gander, J Arch exper Path und Pharmakol 164 324 1932
Gart W Arch exper Path und Pharmakol 201: 207 1943
Gaylord C and H C Hodge Proc Soc Exper Biol and Med 55 46 1944
Gerard R W Arch Neurol and Psychiat 40: 985, 1938
Giaja, J and I N Dmitrijevic Arch internat de Pharmacodyn et Therap 45: 342 1933
Ginsberg A and I Hohlberg Compt rend XI cong internat pharm The Hague 1 559 1913
Githens T S J Exper Med 18: 300 1913
Glasstone S K J Laidler and H Eyring The theory of rate processes McGraw Hill New York 1941
Goldfeder, A Cancer Res 1: 220 1941
Gordon L E and C M Pomerat Proc Soc Exper Biol and Med 50: 202 1942
Gottlieb R. Münch med Wchnschr 61 813 1914
Gray I. E Am J Physiol 84 566 1928
Grosse Brockhoff F and W Schoedel Pflüger's Arch 245 664 1943a Arch exper Path und Pharmakol 201: 417 1943b Arch exper Path und Pharmakol 201: 443 1943c
Grundfest H Am J Physiol 133: P307, 1941
Günther, B and Y Navarro Personal communication 1945
Günther B and J B Odoriz J Pharmacol and Exper Therap 82 303, 1944 J Pharmacol and Exper Therap 83: 1 1945
Gumprecht P Pflüger's Arch 59: 105 1895
Gunn D L Biol Revs 17: 293 1942
Gunn J W C J Pharmacol and Exper Therap 6: 39 1914
Gunn J A Handbuch d exper Pharmakol (Heffter) 5 187 1937
Hale, W U S Hyg Lab Bull no 74 1911

HALL, F G AND R W ROOT Biol Bull 58 52, 1930
HALL, V E AND P E CHAMBERLIN J Pharmacol and Exper Therap 59 451, 1937
HALL, V E, J M CRISMON AND P E CHAMBERLIN J Pharmacol and Exper Therap 59 193, 1937
HAMILTON, J B Yale J Biol and Med 9 327, 1937
HARGER, R N AND H R HULPIEU J Pharmacol and Exper Therap 54 145, 1935
HASKELL, C C Lilly Scientific Bull Ser 1, no 5, p 199, 1914
HAUSMANN, W Pflüger's Arch 113 317, 1906
HEILBRUNN, L V An outline of general physiology Saunders, Philadelphia, 1943
HEMINGWAY, A Am J Physiol 134 350, 1940
HEMMINGSEN, A M Skand Arch f Physiol 46 56, 1925 Skand Arch f Physiol 82 105, 1939
HEMMINGSEN, A M AND A KROGH Publ Health Organ League Nations III 7 CH398 40, 1926
HENDERSON, V E Physiol Revs 10 171, 1930
HENSCHEL, H AND M STEUBER Arch exper Path und Pharmakol 160 401, 1931
HERRMANN, J B J Pharmacol and Exper Therap 72 130, 1941
HERRMANN, L Arch f Anat und Physiol p 64, 1867
HERZOG, F AND H SCHWARTZ Arch exper Path und Pharmakol 151 12, 1930
HILL, A V J Physiol 39 361, 1909
HIRSCHFELDER, A D, J BICEK, F J KUCERA AND W HANSON J Pharmacol and Exper Therap 15 427, 1920
HOAGLAND, H Cold Spring Harbor Symp 4 267, 1936
HOLLINGER, N Abstracts of dissertations Stanford University, California 20 12, 1944-45
HOUSSAY, B A AND C T RIETTI Compt rend Soc de biol 91 27, 1924
HUXLEY, J S Nature 123 712, 1929
HUXLEY, J S AND J F FULTON Nature 113 234, 1924
VON ISSEKUTZ, B Arch exper Path und Pharmakol 78 155, 1914
JACOBJ, C Arch exper Path und Pharmakol 27 119, 1890
JAMIESON, R A J Exper Med 22 629, 1915
JARISCH, A Personal communication cited from Grosse-Brockhoff and Schoedel, 1943c
JENSEN, H Insulin Commonwealth Fund, New York, 1938
JOHLIN, J M Proc Soc Exper Biol and Med 55 122, 1944
JOHNSON, F H, H EYRING AND R W WILLIAMS J Cell Comp Physiol 20 247, 1942
JOHNSON, F H, H EYRING AND W KEARNS Arch Biochem 3 1, 1943
JOHNSON, G E Quart Rev Biol 6 448, 1931
KANITZ, A Temperatur und Lebensvorgänge Borntraeger, Berlin, 1915 Tabulae Biol 2 9, 1925
KNOEFEL, P K J Pharmacol and Exper Therap 84 26, 1945
KOENINCK, A Arch f Anat und Physiol, p 389, 1899
KOSUGE, Y Acta schol med Univ Kyoto 17 361, 1935
KROGH, A Respiratory exchange of animals and man Longmans, Green, London, 1916 Comparative physiology of respiratory mechanisms Univ Pennsylvania Press, Philadelphia, 1941
KUNDE, F Virchow's Arch 18 357, 1860
LAMBRUSCHINI, C Rev Soc argent de biol 14 353, 1938
LANGLOIS, P Arch de physiol 9 152, 1897 Arch de physiol 10 124, 1898
LANGLOIS, P AND C RICHET Travaux du laboratoire Alcan, Paris, 1895
LAUBENDER, W AND H KOLB Arch exper Path und Pharmakol 182 401, 1936
LEBRETON, E Compt rend Soc de biol 116 82, 1934
LEBRETON, E, M NICLOUX AND G SHAEFFER Compt rend Acad Sci Paris 200 1133, 1935
LESER, A J, C H THIENES AND D B TYLER Proc Soc Exper Biol and Med 45 682, 1940

LEBNÉ E AND L DREYFUS Compt rend Soc de biol 64 432 1908
LEWANDOWSKY M Arch f Anat und Physiol p 360 1899
LIBET B J F FAZEKAS, A M MEIROWSKY, E H CAMPBELL AND H E HIMWICH Am. J Physiol 129: 404, 1940
LUCAS, K J Physiol 37 112 1908
LUCHSINGER B Pflüger s Arch 16 510 1878
MCCORMICK N A AND J J R MACLEOD Proc Roy Soc B 98 1 1925
MCELROY, W D J Cell Comp Physiol 21 95 1943
MCGUIGAN R A. J Lab Clin Med 23: 990 1938
MACLEOD J J R. Physiol Reviews 4 21, 1924 Carbohydrate metabolism and insulin Longmans, Green London, 1926
MCQUARRIE I, M R. ZIEGLER AND W E STONE Chinese Med J 58: 1 1940
MAGNE, H. A MAYER AND L PLANTEFOL. Ann de Physiol 8 1, 1932
MANN F C J L BOLLMANN AND T B MAGATH Am J Physiol 68 115 1924
MANSFIELD, G AND Z HORN Arch exper Path und Pharmakol 132 257 1928
MARSH F Trans Roy Soc Trop Med and Hyg 24: 257 1930
MARSON, G M C AND E BELAND Anesthesiology 6 483, 1945
MATISSE G Action de la chaleur et du froid sur l activité des êtres vivantes Larose, Paris 1919
MEYER, H H Arch exper Path und Pharmakol 46: 338, 1901
MORIYAMA H Jap J Exper Med 12 591 1934
MUNCH J C Bioassays Williams and Wilkins Baltimore, 1931
MUTO K. Arch exper Path und Pharmakol 62 494 1910
NEEDHAM J Biochemistry and morphogenesis Cambridge 1942
NEWMAN H W AND A J LEHMAN Arch internat de pharmacodyn et therap 55 440, 1937
NEWMAN, H W AND M L TAINTER J Pharmacol and Exper Therap 57 67, 1936
NICLOUX M. Bull de la Soc chim biol 13 857 1931
NIELSEN E T Acta Med Skand Suppl 90 169 1938
O CONNOR J M Proc Roy Irish Acad 43 23 1936
O CONNOR J M AND D K. O DONOVAN Proc Roy Irish Acad 47: 251, 1942
OKAMURA R Folia pharmacol japon 27: 92 1939
OLMSTED, J M D Am J Physiol 69: 137 1924 Am J Physiol 76 200, 1926
OPPENHEIMER, M J AND A MCCRAVEY Proc Soc Exper Biol and Med 46 513 1941
OZORIO DE ALMEIDA M J Neurophysiol 6: 222 1943 6: 73, 1943
PANELLA A Atti Soc Toscana di Science Nat Mem 24 1, 1907 Cited from Cntbl Physiol 22 512 1908
PATT G H AND J R JOHNSON Am. J Physiol 122: 753 1938
PENHOS J C Rev Soc argent de biol 20: 109 1944
PFEIFFER, C M A FOSTER AND D SLIGHT J Pharmacol and Exper Therap 67 307 1939
PITTINGER, P S AND C E VANDERKLEED J Am Pharm A 4: 427, 1915
PRIBRAM H Temperatur und temperatoren in Tierreiche Leipzig und Wien, 1923
RANDALL, W C Am J Physiol 139 56 1943
RAVENTOS, J J Pharmacol and Exper Therap 64: 355, 1938
REINCKE, J J Deutsch. Arch f klin. Med 16: 12 1875
RICHARDS R K. Anesthesiology 2: 37 1941 Science 97: 313 1943
RICHET C La chaleur animale Alcan Paris 1889
ROTH G B U S Hyg Lab Bull no 102 1916
VAN RYSSELBERGHE P Growth 8 235 1944
SAINT HILAIRE M Travaux du labortoire Ed C Richet, Paris, 1893
SAMOJLOFF A Pflüger s Arch 208: 508 1925
SANNO J Arch exper Path und Pharmakol 65 325 1911
SCHLOMOVITZ B AND C S CHASE J Pharmacol and Exper Therap 8 127, 1916

SCHLOMOVITZ, B AND S A MACHLIS Arch exper Path und Pharmakol 111 1, 1926
SCHOEPFLE, G M AND J ERLANGER Am J Physiol 134 694, 1941
SIEVERS, R F AND A R MCINTYRE J Pharmacol and Exper Therap 59 90, 1937
SIZER, I W Effects of temperature on enzyme kinetics Advances in Enzymology, Interscience, New York 3 35, 1943
SMITH, L W Ann Int Med 17 618, 1942
SMITH, M I AND W T MCCLOSKY Pub Hlth Rep Suppl no 52, 1925
SOLLMANN, T, W L MENDENHALL AND J L STINGEL J Pharmacol and Exper Therap 6 533, 1915
SORDELLI, A, B A HOUSSAY AND P MAZZOCCO Compt rend Soc de biol 89 744, 1923.
SPRINCEANA, C Compt rend Soc de biol 78 224, 1915
STOLAND, O O AND M KINNEY Am J Physiol 49 135, 1919
STORM VAN LEEUWEN, W AND M VAN DER MADE Pflüger's Arch 165 37, 1916
STORMONT, R T AND W E HOOK J Pharmacol and Exper Therap 70 40, 1941
STORMONT, R T, M A FOSTER AND C PFEIFFER Proc Soc Exper Biol and Med 42 56, 1939
SUMNER, F B J Exper Zool 15 315, 1913
SWANSON, E E AND K K CHEN Quart J Pharm and Pharmacol 12 657, 1939
SWANSON, E E, W E FRY AND O W REAGAN J Am Pharm A 34 183, 1945
TAINTER, M L J Pharmacol and Exper Therap 51 45, 1934
TALBOTT, J H New England J Med 224 281, 1941
TIGERSTEDT, R Handbuch der vergleich Physiol (Winterstein) 3 2nd half, 1, 1910
TOURNADE, A, M CHEVILLOT AND G CHARDON Compt rend Soc de biol 128 166, 1938
TRENDELENBURG, P Arch exper Path und Pharmakol 61 256, 1909
TYLER, D B Proc Soc Exper Biol and Med 42 278, 1939 Proc Soc Exper Biol and Med 45 117, 1940
VANDERKLEED, C E AND P S PITTINGER J Am Pharm A 2 822, 1913
VELEY, V H AND A D WALLER Proc Roy Soc B 82 205, 1910
VOEGTLIN, C AND E R DUNN U S Pub Health Rep 38 1747, 1923
WALLER, A D Proc Roy Soc B 81 545, 1909
WEESE, H Arch exper Path und Pharmakol 168 223, 1932 *Digitalis* Thieme, Leipzig, 1936
WEIZSÄCKER, V Arch exper Path und Pharmakol 72 282, 1913
WIDMARK, E M P Biochem Ztschr 282 79, 1935
WIGGERS, C J AND O ORIAS Am J Physiol 100 614, 1932
WINDLE, W F Ann Rev Physiol 5 63, 1943
WINTERSTEIN, H Die Narkose Springer, Berlin, 1919

# LIPINS AND LIPIDOSES

S J THANNHAUSER AND GERHARD SCHMIDT

*Boston Dispensary, the Joseph H Pratt Diagnostic Hospital, and Tufts College Medical School, Boston*

RECENT ADVANCES IN THE CHEMISTRY AND PATHOLOGY OF THE LIPINS (PHOSPHATIDES AND CEREBROSIDES) The pathological chemistry of the phosphatides and cerebrosides is so far chiefly based on observations involving the "chemical anatomy" of these substances rather than their functions in the cells The distribution of these substances in the animal tissues is of great interest for clinical pathology since it has been found that, during certain diseases, specific phospholipids or cerebrosides accumulate in large amounts in organs such as brain, liver, lung, and spleen. In such cases the accumulations of specific lipins are so characteristic that frequently the correct diagnosis is possible only after the chemical identification of the lipins in certain tissues

I CHEMISTRY OF THE LIPINS The customary classification of the lipins into the groups of monoaminophosphatides, (lecithins and cephalins), diaminophosphatides (sphingomyelins), and cerebrosides has to be modified in order to incorporate all lipins known at the present time This classification originated at a period in which it was believed that the various members of each group differed from one another only in the nature of their component fatty acids This assumption, however, holds only for the lecithins, whereas each one of the other groups includes individuals which differ also in the structures of their non-fatty acid components from other individuals of the same group Furthermore, some phosphatides of the acid fast bacteria do not fit into any group of the usual classification

The following scheme contains the lipins known at the present time

1) *Monoaminophosphatides* Fatty acid esters of a phosphorylated polyvalent alcohol, combined with a nitrogen-containing group Their ratio P N is 1 1 (Some monoaminophosphatides contain other organic groups in addition to those already mentioned )
   - A *Lecithins* Phosphoric acid diesters of diglycerides and choline
     *Lysolecithins* Phosphoric acid diesters of saturated monoglycerides and choline
   - B *Cephalins* All known cephalins contain their total nitrogen in form of a primary amino group (ethanolamine or serine)
     - a ) Phosphatidyl ethanolamines.
       Phosphoric acid diesters of diglycerides and ethanolamine
     - b ) Phosphatidyl serines.
       Hydrolysis products *Fatty acids, phosphoric acid, polyvalent alcohols, serine*
2) *Plasmalogens (Acetalphosphatides)* Phosphoric acid diesters of a higher fatty aldehyde acetal of glycerol and of ethanolamine
3) Inositol phosphatides obtained from brain, soybeans and bacteria See text and supplement regarding their composition

4) *Phosphatidic acids, cardiolipins* Hydrolysis products Fatty acids, polyvalent alcohols, phosphoric acids (bound as a monoester)
5) *Phosphatides of acid-fast bacteria* Hydrolysis products Phosphoric acid, polyhydroxy compounds (such as carbohydrates, inositol), fatty acids with straight and branched chains
6) *Diaminophosphatides (Sphingomyelins)* Acid amides of sphingosine with fatty acids (ceramides) in ester linkage with phosphorylcholine
7) *Cerebrosides* Acid amides of fatty acids with sphingosine or dihydrosphingosine in glucosidic linkage with galactose or glucose
8) *Gangliosides* Structure unknown, hydrolysis products sphingosine, neuraminic acid, fatty acids, and galactose or glucose

*Fatty acids in lipins* Since the fractionation of lipins is possible so far only to a limited extent, the analysis of the fatty acid components obtained by hydrolysis of the lipid fractions is at present the only method which permits an insight into the finer composition of the phospholipids isolated from tissues. Because of the probability of a metabolic relationship between the various lipins and the neutral fats, a comparison between the structures of their component fatty acids is of physiological interest in regard to the intermediary metabolism of lipids Comparative studies of the fatty acids occurring in the lipins of various organs are all the more valuable as our knowledge of the enzymes involved in the transformation of fatty acids is as yet too scarce to permit another experimental approach to the intermediary metabolism of the lipids. Systematic investigations of the fatty acid components of phospholipids have been carried out in the laboratories of Anderson (1), Bloor (2), Hilditch (3), Klenk (4, 5), Thannhauser (6) Considering the large number of physiological fatty acids, the complicated possibilities of their isomerism and the technical difficulties of their fractionation, it is obvious that the available data, numerous as they are, permit only relatively few conclusions of a more general physiological interest For this reason the important investigations in this field can be discussed in this article only to a very limited extent

*Some recent advances in the methods of separation and identification of fatty acids* The fractional distillation of fatty acids or their methyl esters under highly reduced pressure has been successfully applied to the separation of small amounts of fatty acid mixtures Special distilling columns for this purpose have been described by several authors (7, 8, 9, 10)

Brown and his co-workers (11) developed the fractional crystallization of fatty acids or their methyl esters from organic solvents at very low temperature This technique is of particular importance for the isolation of unsaturated fatty acids such as linoleic, linolenic, and arachidic acid because it avoids the formation of conjugated double bonds caused by the usual methods of purification

Spectrophotometry in the ultraviolet range has been successfully applied to estimate the percentage of conjugated double bonds in mixtures of unsaturated fatty acids (12)

Some data concerning the infra red spectrum of fatty acids are compiled in the review published in the Analytical Edition of Industrial and Engineering

Chemistry (13) Infra red spectroscopy, however, has not yet been applied to tissue analysis

Analyses of the X-ray diffraction pattern have yielded valuable results concerning the structure of natural higher fats (14, 15)

The investigation of monolayers of fatty acids has led to important conclusions regarding the structure of fatty acids with branched chains (16)

Fatty acids in *animal* lipins Klenk (17) concludes from the results of extensive investigations embracing species of all classes of vertebrates that the ether soluble phosphatides contain fatty acids with even carbon chains of 16 to 22 C atoms whereas acids with 24 C atoms are exclusively found as components of cerebrosides and sphingomyelins The fatty acid mixture obtained from ether-soluble lipins consists of individuals of various degrees of unsaturation. On the average the number of double bonds per fatty acid molecule of the monophosphatide fraction increases with the number of C atoms The composition of the fatty acid mixture obtained from the neutral fat of liver is remarkably similar to that of the monoaminophosphatides The acids of the storage fats, however, differ strongly from those of the monophosphatides inasmuch as the highly unsaturated fatty acids with 20 and 22 C atoms are absent The similarity between monoaminophosphatides and liver triglycerides on the one side and the differences between phosphatides and storage triglycerides on the other side in respect to their component fat acids is interpreted by Klenk as a consequence of metabolic relations between phosphatides and triglycerides in the liver Klenk assumes that the liver is the organ in which the highly unsaturated fat acids are incorporated into the phospholipids A metabolic relationship between fat acids of various chain lengths has become even more plausible since the recent elucidation of the structure of arachidonic acid (18, 19, 20) The formula of arachidonic acid is that of a 5, 8, 11, 14 eicosatetrenoic acid It is interesting to note that the location of the double bonds in the chains of the terminal C atoms of arachidonic and linoleic acids is identical Dolby, Nunn, and Smedley MacLean (18) discuss the possibility that linoleic acid would represent the physiological precursor of arachidonic acid Arachidonic acid could be formed from linoleic acid by condensation of the carboxyl group with a two carbon chain and subsequent desaturation

Arachidonic acid

$$CH_3(CH_2)_4CH\ CHCH_2CH\ CHCH_2CH\ CHCH\ CH\ CHCH_2CH_2CH_2COOH$$

Linoleic acid

$$CH_3(CH_2)_4CH\ CHCH_2CH\ CHCH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$$

It can be gathered from the preceding discussion that the differences between the fatty acid composition of phosphatides, liver fats, and body fats involve mainly the fraction of the highly unsaturated fat acids This is of particular interest inasmuch as Burr (21) discovered some time ago that some of these acids, namely, linoleic and arachidic respectively are "essential fatty acids"

It was emphasized in the introduction to this chapter that, as a rule, it is not yet possible to separate monoaminophosphatides which differ from each other exclusively in regard to their component fat acids Since the purified monophosphatide fractions usually contain saturated and unsaturated acids in approximately equal amounts, it was frequently assumed that the natural monophosphatides contain in each molecule one saturated and one unsaturated fat acid

Lesuk and Anderson (22), however, succeeded recently in isolating a saturated lecithin, dipalmitolecithin, from the ether-insoluble lipid fraction of larvae of cysticercus fasciolaris [1] The insolubility of this natural saturated lecithin in ether is in accordance with the properties of other saturated lecithins obtained by hydrogenation of natural lecithins and of synthetic saturated lecithins (23) Thannhauser and his co-workers (24) encountered saturated monoaminophosphatides in the fraction of the ether-insoluble phospholipids obtained from animal tissues such as brain and lung In view of these findings it is obvious that the classical fractionation of the lipins into an ether-soluble and ether-insoluble part can no longer be interpreted as a separation of mono and diaminophosphatides [2]

*Fatty acids of cerebrosides and sphingomyelins* The component fatty acids of the cerebrosides and sphingomyelins differ from those of the monoaminophosphatides So far, it seems that the latter group of animal and plant phosphatides contains only fatty acids with even numbers of C atoms up to 22 The majority of sphingomyelins and cerebrosides, however, are characterized by component fatty acids with chains of 24 and more C atoms, namely, lignoceric acid, n-tetracosanic acid, $C_{24}H_{48}O_2$ m p 83–84°, cerebronic acid, $\alpha$-hydroxylignoceric acid, m p 100–101°, $\alpha_D^{20}$ (Pyridine) + 3 41°, nervonic acid, 9,10 dehydrolignoceric acid $C_{24}H_{46}O_2$ m p 40–41°, $\alpha$-hydroxynervonic acid $C_{24}H_{46}O_3$ m p 65°, $\alpha_D^{20}$ (Pyridine) + 2 87 The structure of cerebronic acid which was the object of discussion for many years now appears to be definitely established by its synthesis (26)

Until very recently the chain length of the fatty acids isolated as hydrolysis products of these lipins did not seem to exceed 24 C atoms Klenk and Schumann(27), however, succeeded in isolating from the hydrolysis products of a cerebroside fraction a hexacosanoic acid which crystallized from acetone in bright scales and melted at 45–45 5°

*Fatty acids in phosphatides of acid-fast bacteria* Anderson and his co-workers (1) demonstrated in a series of investigations the presence of saturated fat acids with branched chains in the phosphatides and in the acetone-soluble lipids of acid-fast bacilli These substances have lower melting points than their isomers with straight chains and appear usually as liquids at room temperature

*Tuberculostearic acid* (28, 15), 10-methyl stearic acid, $C_{19}H_{38}O_2$, has been obtained in pure form from the phosphatides and the acetone-soluble lipids of

[1] The analysis of this particular tapeworm was undertaken because its larvae produce a sarcomatous growth in the surrounding tissue of the host

[2] The ether fractionation of phospholipids has recently been criticized by Sinclair (25) for other reasons

bercle bacilli and melts at 10–11°C Its optical inactivity is attributed by elman (28) to the remoteness of the asymmetric C atom from the carboxyl oup rather than to racemisation Synthetic dl 10 methyl stearic acid melts . 20–21° The difference in the melting points between natural tuberculo- earic acid and synthetic dl 10-methylstearic acid is not yet definitely explained is possible that the natural compound differs from the synthetic only in its ereoisomeric properties. This view is supported by the results obtained with mparative analyses of the x ray diffraction pattern of both compounds (15) atural tuberculostearic acid produces no specific biological reactions after jection into animals

*Phthioic acid*, $C_{28}H_{56}O_2$, was discovered by Anderson and Chargaff (29, 30) in he phosphatides and acetone soluble lipids of all strains of tubercle bacilli :t is dextrorotatory ($\alpha_D^{20°} = +12.6°$) and doubtlessly represents a fatty acid with branched chains although its structure is not yet known in detail Sten agen and Ställberg (31) conclude from their studies on monolayers of phthioic acid that its structure should be expressed as that of a trisubstituted acetic acid with two long and one short chain. Phthioic acid as well as the phthioic acid-containing phosphatide fractions of tubercle bacilli are of great biological nterest since these substances produce typical tubercular changes after injection nto tissues (32)

*Phytomonic acid* (33), $C_{20}H_{40}O_2$, m p 24°, has been isolated from the phos phatides and the acetone soluble lipids of the crown gall bacillus (phytomonas umefaciens) The low melting point suggests the assumption of a branched hain structure The discovery of a branched chain fatty acid in this micro- rganism demonstrates the fact that this type of fatty acid is not limited to acid-fast bacteria

*Mycocerosic acid*, $C_{30}H_{60}O_3$, m p 27–28°, was obtained by Ginger and Anderson 34) from the wax present in the cell residues from the preparation of tuberculin :t forms an ether soluble lead salt and is levorotatory ($\alpha_D^{20°}$ Chloroform = −5° o 6°)

No fatty acids with branched chains have been found in yeast phosphatides 35) and in tuberculous lung tissue (36)

Recently Weitkamp (37) reported observations suggesting the presence of branched chain fatty acids in wool fat (degras) Weitkamp's conclusions have been confirmed by Velick and English (38) who synthesized d 14-methyl palmitic acid and found it identical with the natural acid obtained from wool

Another group of acids which are very characteristic components of the acid fast bacteria, the *mycolic acids*, (39) are apparently *not* present in the phos- phatides of these micro-organisms. These substances have been discovered by Anderson and his co-workers as constituents of the so-called waxes of the acid fast bacteria and are hydroxyfatty acids with branched chains of very high molecular weights Mycolic acids account for a large part of the lipids of *acid-fast* bacteria They are solid at room temperature and soluble in ether On pyrolysis (heating at 250° to 300° under highly reduced pressure) they decompose yielding fat acids of straight or branched chains For example the mycolic acid obtained from the wax of human tubercle bacteria (40) has the

probable formula $C_{88}H_{176}O_4$, melts at 54–56°C, and is dextrorotatory ($\alpha_D^{20°}$ = +1 8°) The noncarboxylic oxygen atoms are present in the form of one hydroxyl and one methoxyl group On pyrolysis mycolic acid yields n hexacosanic acid

The mycolic acids are of great biological significance being probably responsible for the acid fastness of the mycobacteria

*Glycerophosphate in phosphatides* Theoretically, all phosphatides containing glycerophosphate could be derivatives either of alpha or of beta glycerophosphate No method permits at present the separation or differentiation of these two possible series of phosphatides Some earlier authors attempted to obtain information concerning this structural problem by investigating the mixture of the two glycerophosphates obtained after hydrolysis of natural phosphatides. However, Folch (41) was able to demonstrate that the structure of glycerophosphate obtained by acid or alkali hydrolysis of phosphatides does not permit *conclusions as to the structure of the intact phosphatide molecule since* the phosphate linkage in glycerophosphate undergoes rearrangements on boiling with acids or alkalis Thus it appears that the solution of this structural problem has to await the development of new methods for the splitting of phosphatides, perhaps the use of hydrolyzing enzymes

*Lecithins* The method for the preparation of lecithin (Levene and Rolf, 42) has been simplified by Pangborn (43)

A new lecithin, dipalmitolecithin, has been isolated by Lesuk and Anderson (22) from the larvae of cysticercus fasciolaris With this discovery, a saturated lecithin has been found for the first time in biological material Some properties of this lecithin have already been discussed (p 278)

*Aminoethanol cephalin, phosphatidylethanolamine* This fraction which until recently was considered to be the only constituent of cephalin, has now been obtained reasonably free of other phosphatides by Folch (44) Its components appear to be exclusively fatty acids, glycerol, phosphoric acid, and ethanolamine. In some important properties it differs considerably from the "cephalin" described by earlier investigators Phosphatidylethanolamine from ox brain is easily soluble in alcohol and contains only traces of ash It represents a slightly sticky white powder which, on standing in the dark in an evacuated desiccator for two weeks, acquires a tan color which later turns into a deep brown Its iodine number of 78 suggests the presence of two double bonds for each atom of P

*Phosphatidyl serin* was obtained by Folch (44) from the crude cephalin fraction of ox brain by fractionated precipitation of cephalin solutions in chloroform with alcohol Its components are fatty acids, glycerol, phosphoric acid, and l(+)-serin When the substance is prepared from brain cephalin without acid treatment, it contains approximately 12 per cent of ash, which appears to originate from the salts of the serine phosphatide with potassium, sodium, and calcium The iodine number of 33 would indicate the presence of one double bond per atom of phosphorus According to the description of Folch, phosphatidyl serine seems to be soluble in alcohol to a considerable extent

Chargaff, Ziff and Rittenberg (45) found that amino acid-containing phosphatides are present in the phosphatide fractions of various animal organs such as brain, lung, heart, and liver In brain phosphatides the amino acid nitrogen accounts for approximately 30 per cent of the amino nitrogen, in lung for 23 per cent It is interesting that egg yolk phosphatides are practically free of amino acid nitrogen

*Inositol phosphatides, lipositol* The presence of inositol in phosphatides was first demonstrated in Anderson's laboratory (46, 47) in 1930 in bovine tubercle bacilli, in 1939, Klenk and Sakai (48) reported the isolation of inositol monophosphate from soy bean cephalin after acid hydrolysis, in 1942, Folch and Woolley (49) found inositol in the least alcohol soluble fraction of brain cephalin. According to these authors, the inositol-containing fraction represents approximately one fourth of the total brain cephalin.

By fractionated precipitations of brain cephalin Folch succeeded in arriving at products containing 6.8 per cent inositol These fractions still contained serine as well as glycerol and have certainly to be considered as mixtures.* Woolley (50) prepared from soy bean phosphatides a cephalin fraction with 16 per cent inositol, this fraction which he called lipositol is apparently free of glycerol and serine The hydrolysis of lipositol with acids respectively alkali yielded in equimolecular proportions phosphoric acid, inositol, galactose, tartaric acid, ethanolamine, oleic acid, and a mixture of saturated fatty acids (palmitic, stearic, and cerebronic acid) the sum of which was equimolecular to the amount of oleic acid The phosphoric acid appears to be esterified with inositol which is probably linked to the carbohydrate as galactoside in the lipositol molecule Woolley reported in his paper on soy bean lipositol that inositol phosphatide fractions of brain contained likewise galactose and tartaric acid (50, p 583)

Soy bean lipositol is only slightly soluble in methanol, ethanol, dioxane, glacial acetic acid. It is insoluble in dry petroleum ether, dry benzene, dry ether, or dry chloroform, but soluble in the moist solvents The preparation contains approximately 12 per cent of ash, which is so firmly bound that it cannot be removed by dialysis of a lipositol suspension in hydrochloric acid

No other nitrogen-containing group, besides colamine, has been found among the hydrolysis products of soy bean lipositol In particular amino acid nitrogen was found to be absent Alkali hydrolysis yielded considerable amounts of colamine, while only very small amounts of the base were obtained after acid hydrolysis.

*Acetal phosphatides plasmalogens* This group of phosphatides was discovered by Feulgen during histochemical studies on the staining of tissues by fuchsin sulfurous acid This reagent which produces a purple color with aldehydes permits, after treatment of the cells with sulfuric acid, a selective staining of the chromatin structures of the nuclei due to the aldehyde groups appearing on hydrolysis of thymonucleic acid. Feulgen introduced the term "nucleal" for his staining method He observed very soon that the staining effect of

*See supplement

the nucleal reagent was not strictly limited to the cell nucleus, but inc many tissues the cytoplasm, although with a lesser intensity The ste the cytoplasm was successful only in undefatted slices while that of th was not influenced by any treatment of the tissues with lipid solvents. concluded that different substances were responsible for the staining c and cytoplasma, he proposed, therefore, for the unknown carriers of the sulfurous acid staining in the cytoplasm the term "plasmal" Since already found in his earliest histochemical experiments that the stainin plasma was strongly accelerated and intensified by preliminary treatmer tissue with acid or with mercuric chloride he postulated that the plas not present in the cell as such, but in the form of a hypothetical precurs he designated as plasmalogen

Attempts to isolate plasmal and plasmalogen resulted in the observat these substances were found exclusively in the phospholipid fraction investigated tissues On addition of a small amount of mercuric chl phospholipid suspensions from brain, muscle, or heart, a strong plasmal was immediately obtained Feulgen, Imhauser, and Behrens (51) w to isolate the aldehyde from horsemuscle phosphatides by steam dis and subsequent condensation with semicarbazone as a crystallized semic and to demonstrate that this substance consisted chiefly of palmitic a semicarbazide contaminated with a small amount of stearic aldehyc carbazide An improved method for the preparation of plasmal semic from horsemeat has been reported by Behrens (52) He was able to 1–1 5 gram of plasmal semicarbazide from 10 kgm horsemeat I Anchel and Waelsch (53) isolated fatty acid aldehydes from relativel amounts of tissues The proportion of palmitic and stearic aldehyde in plasmals from different organs and species It is certain that all contain besides palmitic and stearic aldehyde at least one other fatty a which so far could not be isolated on account of the solubility of its semi-ca

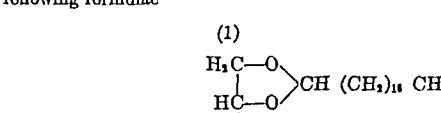
The isolation of plasmalogen was accomplished by Feulgen and Ber on the basis of its relative stability against alkali When the pho emulsion from beef muscle was treated with sodium hydroxide, a cons part of the plasmalogen remained intact while the contaminating phos were saponified From the reaction mixture, plasmalogen could be prec together with the fatty acids by converting them into the brucine salts extraction of the brucine soaps with acetone, the plasmalogen could be by repeated treatment of the acetone-insoluble residue with benzene i dry pure plasmalogen is not soluble The substance is then crystalliz alcohol at room temperature

*Hydrolysis products of plasmalogen* By treatment with mercuric c plasmalogen is split into a higher aldehyde and colamine glycerop (this substance crystallizes in fine needles from alcohol and is very so water, but insoluble in cold alcohol, m p 86–87°) When heated with cent sodium hydroxide for 6 hours, plasmalogen is split into colam plasmalogenic acid which can be isolated as lithium salt Feulgen and

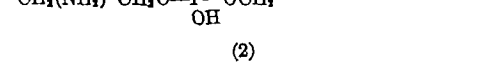
were able to demonstrate that this split-product is composed of glycerophosphate and a fatty aldehyde in equivalent amounts

*Structure of plasmalogen* Feulgen and Bersin concluded from their observations that plasmalogens are acetals of fatty aldehydes with colamine glycerophosphate The structure of stearal plasmalogen would be represented by either of the following formulae

(1)



(2)



The assumption of an acetal linkage is supported by the stability of the nitrogen free group against alkali, its sensitivity against acids and mercuric chloride

Plasmalogen can be determined colorimetrically on the basis of the test with Schiff's reagent (54, 55, 56)

*Properties of acetal phosphatides (plasmalogens)* Plasmalogens have no definite melting point but become soft at 50° and decompose above 150° They are insoluble in water, but soluble in dilute aqueous potassium hydroxide On neutralization of the alkaline solution they precipitate They are sparingly soluble in cold alcohol, but considerably more soluble in hot alcohol Methyl alcohol is a better solvent for plasmalogens than ethyl alcohol They are easily soluble in chloroform but insoluble in ether, petroleum ether, and acetone When an alcoholic solution of plasmalogen is quickly cooled, the acetal phosphatides precipitate as a waxy, amorphous mass, but on slow cooling they come out in the form of globular aggregates which appear under the microscope as composed of long needles

*Phosphatidic acids* The term phosphatidic acid is used for substances which resemble the monophosphatides in their structure and composition, but which differ from these lipins by the absence of nitrogenous constituents

Phosphatidic acids were discovered in cabbage leaves by Chibnall and Channon (57) The free acids are soluble in organic solvents such as ether and acetone, but very slightly soluble in water The sodium salts are soluble in water, but insoluble in ether and very slightly soluble in cold alcohol The barium, calcium, and lead salts are insoluble in water, but easily soluble in ether So far, phosphatidic acids have been mainly found in plants

Plasmalogenic acid, the phosphatidic acid of plasmalogen, has been obtained

by Feulgen and Bersin (51) after saponification of plasmalogen with hot sodium hydroxide

Pangborn (58) reported the isolation of a phosphatidic acid, cardiolipin, from beef heart Its presence seems to be essential for the complement-binding activity of beef heart extracts with sera of syphilitic patients Cardiolipin alone has no complement-fixing power, but mixtures of cardiolipin, cholesterol, and lecithin resemble beef heart extracts prepared for diagnostic use in regard to their serological behavior

The lipid contains phosphorus amounting to 4 1 per cent, fatty acids, and has an approximate molecular weight of 700 Free cardiolipin is very unstable, but its sodium salt is rather stable

The separation of cardiolipin from lecithin was accomplished by the trans formation into a barium salt which is insoluble in alcohol

*Phosphatides of acid-fast bacteria* Our knowledge of this interesting group of lipins is mainly based on the investigations of Anderson and his associates (1) The chemical composition of these phosphatides differs very markedly from that of the phosphatides isolated from animal tissues The fatty acids of bacterial phosphatides have already been discussed Choline and colamine are absent among the hydrolysis products The N P ratio in the phosphatides of these micro-organisms is very much lower than 1 Since these phosphatide fractions have not yet been separated into individual components it is still an open question whether they are to be considered as mixtures of phosphatidic acids with a small amount of monoaminophosphatides or as polyphosphatides containing several atoms of phosphorus per atom of nitrogen The phosphatides from acid-fast bacteria resemble monoaminophosphatides in their solubility They are easily soluble in ether, and they are precipitated from their ether solutions by means of acetone Besides glycerol, these substances contain inositol or carbohydrates in ester linkage with the phosphoric acid group On mild alkaline saponification of the phosphatide fraction of human tubercle bacilli, Anderson, Lothrop, and Creighton (59) obtained a mixture of non reducing organic P esters which could be fractionated to a certain degree After dephosphorylation, mannose, inositol, and glycerol were obtained The hydrolysis of phosphatides obtained from the residues of the manufacture of tuber culin yielded different intermediary phosphoric esters (60) On total hydrolysis glycerol, mannose, inositol, and a glucosazone-forming sugar were found

The phosphatides of phytomonas tumefaciens (61) do not contain carbohydrate components The only organic P ester isolated from their hydrolysis product was glycerophosphate These lipins differ further from those of acid-fast bacteria inasmuch as they contain choline and colamine The N P ratio is approximately 1 1 They differ, however, from animal monophosphatides in their content of fatty acids with branched chains

Anderson and his associates made the interesting observation that the composition of the lipid fractions of micro-organisms which are grown in synthetic media greatly depends on the composition of the medium For example, Creighton, Chang, and Anderson found phosphatides to be absent in the lipids

a strain of human tubercle bacilli cultivated on a modified synthetic Long edrum in which glycerol was replaced by glucose

*Sphingomyelins* Sphingomyelins are phosphatides in which the sphingosine r a closely related base is bound by an NH—CO linkage to a fatty acid, most ommonly to lignoceric acid (n tetracosanic acid) and by an ester linkage to holine phosphoric acid. Klenk isolated pure stearyl sphingomyelin from uman brain in a case of Niemann Pick's disease (63) In the same case liver nd spleen contained a mixture of lignoceryl, nervonyl, and stearyl sphingomyelin 64) Sphingomyelins are insoluble in ether and differ in this respect from the hosphatides of the unsaturated lecithin and cephalin fractions. This property used for the separation of sphingomyelins from the other phosphatides. It ould be emphasized, however, that the insolubility in ether is not a sufficient asis for the identification of a phosphatide fraction with sphingomyelin. phingomyelins are soluble in hot alcohol from which they can be crystallized. hey are soluble in mixtures of chloroform and methanol and in petroleum ther containing 10 per cent methanol Sphingomyelins are almost insoluble acetone and form colloidal solutions with water Sphingomyelins which were iscovered by Thudichum in brain have recently been found in many other rgans (1) and in biological liquids such as blood and milk.

*The structure of sphingosine* The chemical structure of sphingosine has been ucidated by the work of Levene, of Klenk, and of Carter and their associates. ince the fundamental work on sphingosine by Levene and West (65, 66) and apworth (67), its structure has been further clarified in two points 1) Klenk 68) was able to isolate myristic acid, $C_{14}H_{28}O_2$, from the products obtained fter oxidation of sphingosine by means of chromic acid He concluded from is fact that sphingosine contained a straight chain of 18 carbon atoms (not 17 s had been assumed by Levene) 2) Carter and his associates (69) found that enzoyl sphingosine does not react with periodate, but forms a cyclic acetal ith benzaldehyde in the presence of zinc chloride Since periodate is a specific xidant for vicinal glycols and since 1,2 as well as 1,3 glycols form acetals it can e concluded that the two hydroxy groups of sphingosine are present in 1,3 osition. On the basis of these observations the structure of sphingosine can e expressed by the formula

$$CH_3\ (CH_2)_n\ CH\ CH\ CH(OH)\ CH(NH)CH_2OH$$

Since sphingomyelin does not react with nitrous acid it must be assumed that he amino group of sphingosine is linked to one of the other components of the phingomyelin molecule Levene (70) succeeded in isolating lignoceryl hydrophingosine as a product of the partial hydrolysis of hydrogenated sphingomyelin Thannhauser and Fraenkel isolated lignoceryl sphingosine (F.P 90–90.5°) from igs' liver after saponification with methylalcoholic sodium hydroxide (71) Fraenkel and Bielschowsky (72) and Tropp and Wiedersheim (73) succeeded n isolating lignoceryl sphingosine from pigs' liver and beef spleen without reliminary saponification The conditions prevailing throughout the isolation rocedure were so mild that an artificial formation of lignoceryl sphingosine

was excluded Substances resembling lignoceryl sphingosine in their general structure (i e , amides of fatty acids with sphingosine) have been designated as ceramides by Thannhauser and his associates (72) The ceramide group is a constituent of the sphingomyelins as well as of the cerebrosides The isolation of ceramides from tissues suggests that these compounds are formed in the intermediary metabolism of sphingomyelins and cerebrosides

Lignoceryl sphingosine shares with sphingomyelin the inertness against nitrous acid and therefore has to be considered as an acid amide

Thannhauser and Reichel (74) obtained for the first time lignoceryl sphingosine by the enzymatic hydrolysis of sphingomyelin during the incubation with liver extract The only ceramide which has so far been isolated as a split-product of sphingomyelin is lignoceryl sphingosine These authors claimed the existence of a sphingomyelin in which the second hydroxy group would be esterified with palmitic acid In later investigations, it was found that the palmitic acid was not a part of the sphingomyelin molecule, but a part of saturated lecithin which tenaciously adheres to the sphingomyelin fraction (see below)

Ceramides differ from sphingomyelin in their behavior toward ether in which they are soluble They can be recrystallized from hot acetone (75)

*The tests for the purity of isolated sphingomyelin* At present neither sphingomyelin nor any other phosphatide can be reliably identified by determinations of physical constants such as melting points or optical rotation The purity of a phosphatide can therefore be judged only on the basis of the results of its chemical analysis Up to 1937, determination of the N and P contents and the result of Molisch's test for carbohydrates represented the only criteria for the purity of specimens of sphingomyelin Due to the sensitivity of Molisch's test, its negative outcome can be considered as a reliable proof for the absence of cerebrosides The N P ratio, however, is not sufficiently sensitive to permit the detection of contaminating monophosphatides with a desirable degree of accuracy

The introduction of a method for the microdetermination of glycerol into the chemistry of the phospholipids by Blix (76) was therefore a very important addition to the chemical tools available for the examination of the purity of sphingomyelin By the application of this method, Klenk (77) as well as Thannhauser (78) and their associates were able to demonstrate that all specimens of sphingomyelin which had been considered as pure on the basis of the earlier criteria were in fact contaminated with considerable amounts of glycerin-containing phosphatides, probably of saturated lecithins which have many physical properties in common with sphingomyelin, especially the insolubility in ether

The removal of these admixtures proved to be an extremely difficult task which has only recently been accomplished by Thannhauser and Benotti (79) for the isolation of sphingomyelin from lung The isolation of pure sphingomyelin from brain which represents the usual source for the preparation of this lipid has not been accomplished as yet by the exclusive use of fractionation procedures

The difficulties in the purification of brain sphingomyelin arise chiefly from the presence of large amounts of cerebrosides in addition to that of saturated monophosphatides in the crude sphingomyelin fractions Klenk (77) as well as Thannhauser (78) found it necessary to subject the crude sphingomyelin fraction to a treatment with alkali which leaves most of the sphingomyelin intact, but which removes the total amount of monophosphatides by a selective hydrolysis Klenk and Rennkamp (77) refluxed the sphingomyelin fraction with sodium alcoholate Thannhauser and Benotti (79) found that this treatment was too drastic inasmuch as it destroyed a considerable part of the sphingomyelin They achieved the selective destruction of the monophosphatides by shaking the crude sphingomyelin for 24 hours with aqueous N sodium hydroxide at 37°

Nieman (80) recently succeeded in isolating from the hydrolysis products of brain protagon an ether of sphingosine with a tetradecylic alcohol Nieman's findings recall the old observations by Thudichum who found among the hydrolysis products of sphingomyelin an alcohol with 18 carbon atoms which he called sphingol Since none of the later investigators confirmed this result of Thudichum this alcohol received no further attention for a long time The isolation of Nieman's sphingosine ether makes it evident that our present classification of the sphingosine-containing constituents of brain is not yet complete

*Cerebrosides* According to the customary definition, cerebrosides are considered as galactosides of ceramides. The individual cerebrosides were assumed to differ from each other only in respect to the fatty acid components (see page 278) This definition can no longer be considered adequate 1) Lesuk and Anderson (22) isolated from the larvae of cysticercus fasciolaris dihydrophrenosine whose nitrogen-containing component was dihydrosphingosine Carter and Norris (81) found dihydrosphingosine among the hydrolysis products of the cerebrosides prepared from beef brain and beef spinal cord 2) Several investigators (82, 83, 84) found that the cerebrosides which accumulated in the spleens of patients with Gaucher's disease contained as carbohydrate component glucose instead of galactose In consequence of these results Klenk and Rennkamp (85) reinvestigated the cerebrosides of normal tissues They found that the cerebroside fractions of all investigated organs contained small amounts of glucose Thus there is little doubt that glucosidocerebrosides are normal cell constituents as well as galactosidocerebrosides 3) There is evidence of the presence in tissues of a new group of phosphorus-free carbohydrate-containing lipins, the gangliosides, which differ in their chemical structure from the cerebrosides. E Walz (86) described in 1927 the presence in beef brain and beef spleen of a cerebroside fraction which differed from the known cerebrosides by the purple color of the Bial test and by its high sensitivity against acids. After heating for a short time with 10 per cent sulfuric acid the formation of a black flocculent precipitate of humin substances was observed The observations of Walz on normal brain were later confirmed and extended by Blix (87) who found that the new cerebroside fraction gave a positive test for hexosamines with Ehrlich's dimethylaminobenzaldehyde reagent

In 1935 Klenk (88) reported that relatively considerable amounts of similar

substances could be found in the crude sphingomyelin fractions isolated from the brain in cases of Niemann-Pick's disease According to Klenk (89) very large amounts of this fraction accumulate in the brain in Tay-Sachs' disease (infantile form of amaurotic idiocy) The normal cerebrosides are totally replaced by this fraction

*Neuraminic acid* In 1941, Klenk (90) succeeded in isolating a hydrolysis product of the new cerebroside fraction in crystallized form This hydrolysis product for which Klenk introduced the name neuraminic acid represents the group responsible for the characteristic color reactions of the fraction and for its charring on treatment with dilute mineral acid According to the results of the elementary analysis the substance has either the formula $C_{10}H_{19}NO_8$ (molecular weight 281 16) or $C_{11}H_{21}NO_9$ (molecular weight 311 24) It titrates as a monobasic acid and contains its nitrogen in form of a primary amino group It is levorotatory in aqueous solution ($\alpha_D = -54$ 91°) The substance decomposes at 200° without melting point It is easily soluble in water, only little in methyl alcohol, and insoluble in ethyl alcohol and ether

On being heated with dilute mineral acids, the solution soon acquires a brown color During prolonged heating, a black flocculent precipitate appears The substance, however, is stable against boiling at neutral or alkaline reaction

Neuraminic acid does not reduce alkaline copper solution It gives a positive ninhydrine test On boiling it with Bial's reagent an intense red color develops After heating it with Ehrlich's reagent in a paraffin bath of 140°, a strong red color appears The test is strongly positive with 60γ of the neuraminic acid

*Lipoproteins* (91, 92) It has long been known that the extraction of lipins, especially phospholipids, from animal tissues can be carried out successfully only after the denaturation of the proteins The preparation of phosphatides from egg yolk offers a classical example for this observation On exhaustive treatment with ether, only traces of phosphatides go into the organic solvent although the neutral fats can be extracted almost completely in this manner When the yolks are heated with alcohol, however, the total amount of the phosphatides goes quantitatively into the solution and can be easily separated from the protein coagulum

It is possible to extract from egg yolk with dilute aqueous salt solutions undenatured protein fractions with surprisingly high lipid contents This fraction can be precipitated and redissolved without much change in its lipid content or its solubility

Some of the lipid protein complexes migrate in the electrical field like homogeneous substances (93) In order to separate the lipins from their protein carriers, boiling with alcohol or other denaturing procedures are required while the extraction with ether without a preceding denaturation of the proteins does not lead to the dissociation of the lipid protein complex.

It is difficult to reconcile these observations with the assumption that the lipoproteins represent accidental mixtures of lipids and proteins rather than chemical lipid protein complexes On the other hand, no investigator has succeeded as yet in isolating a lipid protein complex in which the lipid fraction

was homogeneous The lecithovitallin from egg yolk which has recently been reinvestigated by Chargaff (94) contained approximately equal amounts of lecithin and cephalin

Liponucleoprotein complexes recently isolated by ultracentrifugation from extracts of normal chicken embryos and from infectious extracts of Rous chicken tumor I contained likewise mixtures of various lipids (95, 96) The same holds true for a lipoprotein with thromboplastic effects isolated from lung tissue (92, 93, 94)

The heterogeneity of the lipid components renders it very difficult to attempt a chemical interpretation of the experimental observations. In this respect, the chemistry of the lipoproteins faces a much more complicated problem than that of many other conjugated proteins such as nucleo-, chondro-, or chromoproteins which are characterized by homogeneous prosthetic groups Additional difficulties are caused by the great instability of the lipoproteins which has so far prevented any attempt of a finer fractionation of the crude products. It is obvious that, under these circumstances, the experimental facts do not as yet provide a basis for a discussion concerning the forces responsible for the associations between lipins and proteins in the complexes described in this chapter

II Recent aspects of the physiology of the lipins In the studies of the past few years, the chemical functions of the phospholipids in metabolism and nutrition have received more attention than the biological significance of their physicochemical properties for cell permeability and other functions of the cell membrane (1)

Sinclair's investigations (2, 3, 4, 5), especially his observations on the rapid incorporation of ingested elaidic acid (fed as elaidin) into the phospholipid fraction of organs, suggest that the phospholipids have an important rôle in fatty acid transport and fat metabolism Artom and his associates (6, 7) came to similar conclusions on the basis of their observations that the injection of iodinized fatty acids was followed by the appearance of iodinized fatty acids in the phospholipid fractions of liver and red globules In addition, Artom (8) found a very considerable temporary increase in the amount of liver phospholipids in dogs which had been fed with large quantities of neutral fats.

Phosphatides are possible precursors of choline and therefore are at least indirectly connected with its functions such as the transmethylation and lipotropic action Investigations by Engel (9) Stetten and Grail (10), and Fishman and Artom (11) have demonstrated that the amounts of phospholipids, especially those of choline-containing phospholipids, are considerably diminished in choline-deficient rats, the addition of choline to the diet compensates this effect in very young but not in older animals Fishman and Artom assume that choline deficiency is not the only factor responsible for the decrease of liver phosphatides in rats under experimental diets According to these authors, the response of very young animals to choline can be explained by the assumption that weanling rats have a sufficient stock of the hypothetical additional factors

Similarly the metabolism of cephalin might be involved in that of its compo-

nents such as colamine and inositol It was demonstrated by Stetten (12, 13) that colamine in the presence of methyl donors, especially methionine, prevents the development of fatty livers Fishman and Artom (11) found, however, that the addition of colamine or other non-choline substances with lipotropic activity to a choline-deficient diet had no effect on the amounts of liver lecithin although it increased those of the non-choline phospholipids

The marked lipotropic effect of inositol has been observed in several laboratories (14)

Beveridge and Lucas (15) pointed out that it is difficult to interpret the influence of the diet on the amount of the total liver phosphatides due to the considerable variations of the chemical composition of liver tissue They found that neither the removal nor the addition of choline, inositol, corn oil, or of the non-essential fat from the diet affected the total relative amounts of phospholipids in rat liver if the calculation was based on the dried defatted liver tissue These observations, however, would have no bearing on the influence of dietary constituents on the mutual proportion of choline-containing phosphatides and cephalins

Much of the evidence obtained in experiments on intact animals supports the view that the digestive tract and the liver represent the most important organs in the metabolism of the phospholipids The relatively high phospholipid content of the lung has led to the question if this organ has an essential function in the lipid metabolism of the organism MacLachlan (16) has studied the problem on fasting mice in which a rapid mobilization of fats takes place No significant change in the phospholipid content of the lungs was observed under these conditions On the other hand, there is evidence that the formation of phospholipids is not limited to the specific activity of liver and intestinal tissue, but represents a more general cell function It has been demonstrated (17) that a number of isolated tissues (brain, kidney, liver, intestines) incorporate radioactive phosphorus into their phospholipids

*Influence of hormones on the metabolism of phospholipids* The removal of the hypophysis and thyroid glands in dogs is accompanied by a considerable increase of the amount of all blood lipids (18)

Entenman, Lorenz, and Chaikoff (19, 20) demonstrated that the injection of estrogens into male or female immature chickens causes a large increase of all blood lipids This effect is undoubtedly the main reason for the increase of the blood phosphatides already observed by earlier authors (21, 22) in the laying hen Since it could be demonstrated (23) that liver slices from birds which had received estrogens are capable of incorporating radiophosphorus at a higher rate into their phosphatide fraction than slices from untreated birds, it would appear that the liver is the site of the increased phospholipid formation in laying birds

The phospholipid contents of the pig in various stages of its embryonic development have been studied by Gortner (24) He observed a steady decrease of the amounts per gram of dry weight during the gestation This behavior differs from that of the rabbit embryos (25) in which a strong increase of the phospholipids was found during the development

*Experiments with $P_{32}$* Since recent comprehensive reviews of this field have been given by Hevesy in Annual Reviews of Biochemistry (1940) (26) and by Chaikoff in this journal (1942)(27), only investigations which have since been carried out will be discussed in this article

Investigations by Fishler, Entenman, and Chaikoff (28) furnished strong evidence that the liver represents the only site of formation of the plasma phospholipids These authors administered radioactive sodium phosphate intraperitoneally to hepatectomized dogs It was found that the amounts of radioactive plasma phospholipids at several intervals up to 6 hours after the injections was only very small in comparison to those found in the control animals. Despite their inability to form plasma phosphatides, the hepatectomized animals were still capable of synthesizing phosphatides at a normal rate in kidney and intestines

Interesting new observations concerning the fate of the plasma phospholipids were obtained by Zilversmit, Entenman, Fishman, and Chaikoff (29) and by Reinhardt, Fishler and Chaikoff (30) The first group of investigators found, in agreement with earlier observations by Hahn and Hevesy (31), that radioactive phospholipids, when injected intravenously, are rapidly and to a large part removed from the plasma The larger part of the injected plasma phospholipids could be recovered from various tissues such as liver, spleen, intestines, kidney, and red globules The second group of authors found that a portion of the radioactive phospholipids reaches the lymphatic channels and was found in the lymph of the thoracic duct The high rate of the passage of the phospholipids from the bloodstream through the tissues to the lymph channels and back to the bloodstream leaves no doubt that the phospholipids permeate the capillary membranes as such Since the phospholipids which passed into the thoracic duct are returned to the blood, the observations discussed above establish the existence of a partial "internal circulation of the phospholipids." Apart from the physiological significance of the passage of the phospholipids through the capillaries it appears that the possibility of such an internal circulation must be generally considered in the interpretation of results obtained by the use of tracersubstances in intact animals

Patterson, Keevil and McHenry (32) found that in choline-deficient rats the turnover of phospholipids was smaller than in normal animals These results substantiate the conclusions reached by Stetten and Grail (10) on the basis of phospholipid determinations in livers of choline-deficient rats

As a general summary of the progress achieved in the physiology of phospholipids by the use of tagged molecules it can be said that tracer substances were used like tracer bullets in order to lighten the pathway and the location of the tagged substances in the organism However, the studies with $P_{32}$ have not so far contributed to our knowledge of the chemical mechanism of the intermediary metabolism of the phospholipids This will be accomplished only by the chemical isolation of the intermediary products

*Enzymatic hydrolysis of monophosphatides* Our knowledge of the phospholipid-splitting enzymes is still rather fragmentary The available data concern mainly the enzymes involved in the hydrolysis of lecithin.

In analogy with other enzyme reactions, the hydrolysis of the four ester linkages of lecithin is achieved by different specific enzymes It has been found that the enzyme systems—and, in consequence the intermediary pathways—of the breakdown of lecithin are different in different organisms

1) *The lecithinase of clostridium Welchii (33)* This microorganism contains an enzyme capable of hydrolyzing the linkage between the glycerol and the phosphoric acid groups of lecithin Its action results in the formation of phosphoryl choline and diglycerides from lecithin The enzyme is activated by calcium ions and inhibited by phosphate, fluoride, and citrate ions It appears to be identical with the alpha toxin which is responsible for the hemolytic and necrotic effects of type A culture filtrates

2) *Lecitholipases* a) Enzymes liberating *one* of the fatty acid groups Lecitholipases which are capable of hydrolyzing one of the fatty acid ester linkages have been found only in the venoms of various snakes and insects (34, 35, 36, 37) The action of these enzymes leads to the formation of lysolecithins which contain only one fatty acid It seems that these enzymes hydrolyze in preference the ester bond of unsaturated fatty acids in the lecithin molecule

Slotta and Fraenkel-Conrat (38) reported the crystallization from rattle snake venom (Crotalus T Terrificus) of a protein possessing the hemolytic and neurotoxic effects in the same quantitative proportions as the crude venom It can be assumed that this protein is identical with the lysolecithin-forming enzyme Fairbairn (39) developed a method for the quantitative determination of the lecithin-hydrolyzing enzyme present in the venom of the cotton mouth moccasin (Agkistrodon Piscivorus L ) He found, in addition, that the enzyme acts only on lecithin and cephalin, while cerebrosides, sphingomyelin, acetal phospholipids and lysophosphatides are completely resistant

b) Enzymes liberating both fatty acid radicals from lecithin Schmidt, Hershman, and Thannhauser (40) isolated $\alpha$ glyceryl phosphoryl choline from beef pancreas which had been incubated for 3 hours at 37° The simultaneous disappearance of a large part of the preformed phosphatides suggested the assumption that the isolated diester originated from the enzymatic hydrolysis of lecithin In 1935 Contardi and Ercoli (41) obtained by incubation of lysolecithin with rice bran a product which apparently consisted of glyceryl phosphoryl choline in impure form (See supplement )

Pure $\alpha$-glyceryl phosphoryl choline is soluble in water and alcohol, but insoluble in acetone, ether or petroleum ether The substance isolated by Schmidt, Hershman, and Thannhauser is levorotatory ($\alpha_D^{20}$ — 4 8) One of its most characteristic chemical properties is the great lability of the choline ester linkage When the substance is heated in N HCl on a water bath for 30 minutes, it is practically completely hydrolyzed to choline and glycerophosphoric acid In this respect $\alpha$-glyceryl phosphoryl choline closely resembles lecithin whereas phosphoryl choline is very resistant toward acids (42)

The formation of $\alpha$-glyceryl phosphoryl choline is effected by the action of specific intracellular lecitholipases These enzymes appear to be firmly bound in the cells since aqueous tissue extracts contain only small fractions of the

enzymatic activity found in suspensions of minced tissues Pancreatic lipase or duodenal juice are without effect on lecithin or cephalin

Glyceryl phosphoryl choline is hydrolyzed by alkaline phosphatase, but the rate of hydrolysis is approximately 150 times slower than that observed with glycerophosphate as substrate

At present, glyceryl phosphoryl choline represents the only substance which has been identified as an intermediary product of the mammalian metabolism of lecithin. Evidence has been obtained that glyceryl phosphoryl choline is found not only in the pancreas but also in other organs, especially in the mucosa of the digestive system It can be assumed that glyceryl phosphoryl choline is the intermediary substance of lecithin metabolism, especially of its enzymatic disintegration in the intestinal mucosa. On the other hand, the assumption that glyceryl phosphoryl choline might be resynthesized to lecithin in the organism would furnish a simple explanation for the rapid incorporation of ingested fatty acids into the phosphatide fraction (2, 3, 4, 5)

*The determination of lipins in tissues* The extraction of the wet tissue according to Bloor (43) is still the basis of the determination of the total lipins and of the individual lipin fractions This precedes all other analytical procedures For the determination of the total phospholipids the alcohol-ether extract is evaporated to dryness under reduced pressure or in an atmosphere of $N_2$, the dry residue is extracted with a small amount of petroleum ether from which the phospholipids can be precipitated by acetone and magnesium chloride and determined oxydimetrically after treatment with chromic acid The reviewers prefer to determine the total P directly in the petroleum ether extract because the results obtained with this technique are not influenced by the presence of cerebrosides. Gortner (44) recently came likewise to the conclusion that the results obtained with P determination are more consistent than those obtained with the oxydimetric method

Folch and Van Slyke (45) described a different extraction procedure for the plasma lipids by which certain disadvantages of Bloor's procedure (such as the contamination of the final petroleum ether extract with non lipid substances) can be avoided The lipids are precipitated together with the proteins by colloidal iron in the presence of magnesium sulfate Finally, they are extracted from the washed precipitate by an alcohol-ether mixture at room temperature

Various analytical procedures for the quantitative estimation of the individual phosphatide fractions have been devised When they are combined with the determination of the total phospholipids a fairly complete quantitative partition of a given phospholipid mixture is obtained It should be pointed out, however, that the results obtained with different schemes of the partition show some discrepancies which cannot be explained completely as yet One of the difficulties encountered in this field arises from the necessity of hydrolyzing the lipids prior to the chemical analysis. Despite the fact that the general behavior of the lipins toward hydrolyzing agents is fairly well known the conditions required for the quantitative hydrolysis of individual components of the various fractions have not as yet been studied sufficiently to exclude errors due to incomplete hydrolysis

1) The amount of amino nitrogen in phospholipid mixtures corresponds to the total amount of cephalins The Van Slyke method is therefore widely used for the determination of cephalin in phospholipid mixtures It must be emphasized, however, that unsaturated fatty acids evolve considerable amounts of inert gas with nitrous acid (45) Folch, Schneider and Van Slyke (45) found only very small amounts of cephalin in the serum lipids when they used the precaution of hydrogenating the lipids before the determination of the amino nitrogen Entenman and Chaikoff (47) obtained similar results by means of choline determinations in the plasma lipids It appears that amino nitrogen determinations in lipid mixtures should be carried out after the removal of the fatty acids by a suitable method of hydrolysis

2) A micro method for the determination of the two known component nitrogen groups of cephalin, namely, ethanolamine and serine, has recently been reported by Artom (48) It is based on the quantitative liberation of the nitrogen of either substance in the form of ammonia during the oxidation with periodate When this method is combined with the quantitative separation of both substances by the selective adsorption of colamine on permutite the amounts of either substance can be determined In many tissues the sum of ethanolamine and serine as determined according to Artom's method agrees with the values for the total cephalin obtained by other methods In some organs such as brain, kidney, and lung the figures obtained with the periodate method are too high (probably due to the presence of sphingolipids in considerable amounts) It should be mentioned that Artom found considerable amounts of cephalins in blood plasma, contrary to the results obtained with some techniques reported above (See supplement )

3) The sum of the choline-containing phospholipids (lecithins and sphingomyelins) can be estimated by the determination of the choline obtained after refluxing the lipid mixture for 2 hours with saturated barium hydroxide (49) The best method for the determination of choline appears to be the spectrophotometry of its reineckate in acetone solution at 327m$\mu$ (49) Amounts between 50 and 400$\gamma$ can be determined with an error of 5 per cent

Taurog and his co-workers (50) achieved the separation of the choline-containing from the non-choline-containing phosphatides of liver by adsorption of the total phosphatides on magnesium oxide and selective elution of the choline-containing phospholipids by methanol

4) The sum of the glycerol-containing phosphatides (lecithin and glycerol cephalins) is obtained by glycerol determinations in the dried phospholipid fractions according to Blix (51) This method is analogous to the micro methoxyl determination of Vieböck and Brecher (52)

Attempts have been made to determine the glycerol in lipid extracts by periodate oxidation (53, 54) The application of this principle to the analysis of fats appears to give reliable results In the case of phospholipids, the use of periodate for the analysis of glycerol incurred serious difficulties 1) the presence in the hydrolysate of interfering substances such as colamine and serine, and 2) the fact that the usual procedures for the hydrolysis of phospholipids do not lead to the

liberation of glycerol as such, but to the formation of a mixture of $\alpha$ and $\beta$ glycerophosphoric acid, the latter does not react with periodate and thus escapes determination It should be emphasized that $\alpha$ and $\beta$ glycerophosphate, rather than glycerol, should be used as test substance whenever procedures for the determination of glycerol in phospholipid mixtures are to be checked.

5) A convenient micro method for the quantitative partition of phospholipid mixtures into monophosphatides and sphingomyelin has been developed by Schmidt, Benotti, and Thannhauser (55) When lecithin or the cephalins are incubated with N potassium hydroxide at 37° for 15 hours the total amount of their phosphorus groups becomes soluble in dilute acids, while the phosphorus of sphingomyelin remains insoluble under these conditions.

6) A direct microdetermination of sphingomyelin as acetone insoluble reineckate has been described by Thannhauser, Benotti and Reinstein (56) The sphingomyelin reineckate includes that of hydrolecithins which have recently been found to be present in several tissues such as brain and lung

7) A method for the micro determination of cerebrosides has been developed by Brückner (57) It is based on the colorimetric determination of galactose by means of orcinol and sulfuric acid in the hydrolysate of the lipid extracts In regard to its specificity it is superior to the earlier reductometric methods (58), it should be kept in mind however, that the figures obtained by Brückner's method do not include the glucose-containing cerebrosides recently found in Gaucher organs and also in normal tissues. (See supplement )

*Remarks* It can easily be seen that by a suitable combination of several of the procedures just discussed, it will be possible to achieve a rather complete quantitative partition of the phospholipids It must be emphasized, however, that the accuracy of figures obtained by difference should be carefully examined in each case In the majority of tissues, only the total lecithin and the total cephalin fractions are so large that errors of the individual determinations do not seriously interfere with the calculation of the differences

Representative figures of the concentration of phospholipids in various tissues (48 59, 60) and in isolated nuclei (61) have been reported by several authors

Peters and Man (62, 63) have recently reported their experiences concerning the amounts of blood phospholipids in humans under normal and pathological conditions They found that the figures for the phospholipid P in normal serum range between 6 1 and 14 5 mgm per 100 cc High values are often observed in thyroid deficiency and low values in hyperthyroidism although normal concentrations may be found in either condition In patients with kidney diseases the concentration of phospholipids in the serum is usually elevated In both groups of diseases, the ratio between the amount of cholesterol and phospholipids is not altered in comparison to that found in normal serum.

III Lipidoses. The group of metabolic disorders designated as "lipidoses" is a heterogenic collection of disorders To combine this group under the name "lipidoses" has been generally accepted since fat-like substances of different chemical constitution and properties are found which accumulate in reticulum cells and histiocytes The mechanism leading to such an accumulation of lipids

within these cells has not been definitely ascertained In explaining this mechanism theories have been advanced which are based on the faculty of reticulum cells and histiocytes to take up lipids from the blood stream as well as to synthesize and retain these substances within the cell

*A Xanthomatoses* For the determination of cholesterol and its esters in body tissues and serum, the digitonin precipitation of free cholesterol (Windaus, 1) is used On the basis of such a precipitation a method has been described by Schönheimer and Sperry whereby only small quantities of material are necessary (2) This method is generally used for the estimation for cholesterol and its esters in tissues and serum The normal serum values in adults range between 190 and 240 mgm per cent for total cholesterol, between 30 and 60 mgm per cent for free cholesterol, and between 100 and 180 mgm per cent for cholesterolesters The cholesterolesters should constitute between 60 and 70 per cent of total cholesterols in serum

The foam cell, the cholesterol imbued reticulum cell, characteristic of the *various types of xanthomatosis may originate*

(I) From an increased uptake of cholesterol from a serum in which cholesterol is pathologically accumulated, or

(II) From an increased synthesis of cholesterol within the cell without increased supply from the blood stream

I In basing the origin of the foam cell on the increased uptake of cholesterol in the reticulum cell, it is presumed that the cholesterol content of the supplying tissue fluids (blood serum or local tissue fluids) is increased

*An accumulation of serum cholesterol (Hypercholesteremia) may result from* a ) A decreased destruction of cholesterol Thus far no enzyme capable of splitting the terpenlike ring-system has been isolated from mammalian tissues The chemical changes, which occur in the intermediary metabolism of the cholesterol molecule take place in the side chain of that molecule, such as esterification of the alcoholic hydroxyl group, hydrogenation and oxydation on the sterol ring as well as of the side chain It is not definitely known whether the sterol-sex hormones are metabolites of cholesterol or the result of sterol synthesis Cholesterol derivatives in minimal quantities have been isolated from animal organs It was suggested that cholestenone was present in deposits of cholesterol in the arterial wall (3, 4) Hydrocholesterol was isolated from the liver and from the serum of pregnant mares (5) Dicholesterylether is present in the spinal cord of the ox (6) Cholestenone resulted from the action of proactinomyces upon cholesterol (7)

A destruction of the cholesterol skeleton was concluded from negative cholesterol balance experiments (8, 9, 10, 11, 12, 13) In these balance experiments cholesterol was determined as cholesterol-digitonid From the fact that the expected amount of cholesterol did not precipitate with digitonin it cannot be concluded that the cholesterol skeleton was destroyed in the intermediary metabolism The sterol nucleus may well be intact while slight oxidative or reductive changes may have resulted in a sterol unprecipitable with digitonin or not giving the Liebermann-Burchard test Since it has been shown that

bacteria present in the intestinal tract transform cholesterol to a sterol not precipitable by digitonin (14, 15), it may be concluded that the deficit of cholesterol in balance experiments in animals and in men is due rather to bacterial action in the intestines upon cholesterol than to a disintegration of the sterol ring in the intermediary metabolism On the basis of our present knowledge an accumulation of cholesterol in the blood serum or in tissues cannot be explained by a decreased destruction of the sterol ring in the intermediary metabolism

b) The *accumulation of cholesterols* in the organism may be due to an *impaired excretion* of this substance Such a disturbance with resulting retention of cholesterol may be functional or mechanical As far as we know cholesterol is excreted by the liver via the bile capillaries (16, 17) and probably also by the cells of intestinal mucosa (18, 19, 20) A disturbance of such an excretory function may result in cholesterol retention in the serum It is not likely that at the onset of a functional disturbance anatomical changes of the affected organ are detectable A diminution of cholesterol excretion may be limited to cholesterol alone, it may also be accompanied by a diminution of other bile constituents like bilirubin and bile acids, i.e , it may occur with or without jaundice (Hypercholesteremia in hepatitis, essential xanthomatosis of the hypercholesteremic type, see p 299)

c) It is evident that *mechanical obstruction* of the bile passages, due to stone, inflammatory or tumerous obstruction, results in a retention of all bile constituents and consequently cholesterols The serum of patients suffering from mechanical obstruction of the bile ducts does not usually show extremely high cholesterol figures (usually twice that of normal) The ratio of cholesterol cholesterolesters (increase of free cholesterol, decrease of cholesterolesters) is only altered if acute or chronic damage of the liver cells accompanies the mechanical obstruction (21, 22, 23, 24) (Hypercholesteremia in obstructive jaundice )

d.) The cholesterol content of the blood serum may be increased without functional or mechanical impairment of cholesterol excretion in cases where *hyperlipemia* (*creamy serum*) occurs Hyperlipemia may be the result of a disturbed metabolism of neutral fat or may be due to an impaired transportation or deposition of neutral fat Whenever neutral fat increases in the serum, cholesterol accompanies the neutral fat and results in an increase of free and ester cholesterol in the serum In such cases the appearance of foam cells is due to an increased uptake of cholesterol from the serum and is observed in different organs especially in skin, spleen, liver and lungs Secondary hyperlipemia with xanthomatosis due to diabetic hyperlipemia (25, 26, 27) Secondary hyperlipemia with xanthomatosis in chronic pancreatitis (28, 29) Secondary hyperlipemia with xanthomatosis in glycogen storage disease (Von Gierke's disease) (30, 31, 32) Idiopathic familial hyperlipemia with secondary xanthomatosis (33, 34, 35) Hepatosplenomegaly with hyperlipemia and xanthomatosis type Bürger Grütz (36)

e) In cases of *hypothyroidism* the cholesterol content of the serum is high The reason for the increase of serum cholesterol is not known Thyroid medication reduces the cholesterol level of the serum In rare cases of hypothyroidism

with very high serum cholesterol levels, foam cells with xanthoma formation may originate in the skin (37)

f ) A local accumulation of cholesterol with foam cell formation may occur in any type of *organs where cells undergo destruction* by inflammatory processes or tumor growth In such circumscribed areas the cholesterol derived from necrotic tissue may appear in the detritus as cholesterol crystals or may be taken up by reticulum cells, thereby changing their appearance to foam cells (Xanthoma cells in inflammatory tissue, osteomyelitis, osteitis fibrosa cystica dis seminata, xanthomatous transformation of the mesentery, xanthoma cells in tumors ) (38)

II *The second mechanism in the origin of xanthoma cells* is probably initiated by intracellular disturbance of enzymes concerned with the cholesterol metabolism As already mentioned enzymes capable of splitting the sterol skeleton are not known to be active in the intermediary and cellular metabolism of animals For this reason an accumulation of cholesterol due to an enzymatic disturbance is not likely to be the result of a decreased cholesterol catabolism, but rather to an increased anabolism, i e , synthesis of cholesterol It has already been shown in the earlier investigations of cholesterol metabolism that the sterol-ring system is constantly synthesized in the mammalian organism (39, 40, 41, 42) Schönheimer, feeding materials containing deuterium, demonstrated that the small molecules, of 2 and 3 carbon atoms, which may be derived from all three food constituents (proteins, carbohydrates and fats) are the basis of cellular sterol synthesis (43) From the experiments of Bloch, using labeled acetic acid, it is evident that considerable quantities of cholesterol are synthesized from a molecule as small as acetic acid (44) The question arises which organs and which cells are capable of cholesterol synthesis This question cannot be definitely answered It is probable that every growing cell during maturation is capable of synthesizing cholesterol but this function seems to be maintained to a higher degree in reticulum cells in which the functional possibilities of em bryonal cells especially of embryonal fat cells to form all kinds of lipids are preserved (45, 46) In the fully developed organism the liver seems to have a special part not only in the excretion but also in the performance of the synthesis of the sterols The experiments of Thannhauser, Enderlen and Jenke on dogs with bile fistulas demonstrated that the synthesis of the sterol-skeleton of bile acids occurs as a biological synthesis in the liver (16) In further experiments (47) it was shown that after liver extirpation in dogs the serum cholesterol is not decreased after 24 hours The lowest values of cholesterol, however are found in the serum of patients suffering from acute yellow liver atrophy (48) This observation supports the theory that the liver plays an important part in the formation of cholesterol in the mature organism It is suggestive to draw an analogy of the behavior of cholesterol and of porphyrins in the metabolism Both substances consist of ring systems, a terpen ring on the one hand and pyrrolrings on the other hand Both ring systems are formed in the cells, but neither the terpenring nor the pyrrolring is split by enzymatic disintegration in animals The mammalian organism is apparently fit to synthesize ring systems

but is not capable of splitting these ring systems after they are formed The enzymatic synthesis of sterols and pyrrols is apparently performed by the same organ cells, namely, the reticulum cells and histiocytes Since the greatest accumulation of reticulum cells is found in the liver, it seems understandable that this organ is the outstanding organ involved in the formation of cholesterol The reticulum cells dispersed in other organs are supposed to be capable of performing the same functions but the actual magnitude of their part in cholesterol synthesis under normal conditions is not well known. We only are aware that the cholesterol content of the serum in acute yellow liver atrophy is markedly decreased but nevertheless cholesterol in the serum is always present indicating that cholesterol is also formed outside of the liver For these reasons different clinical syndromes may occur under pathological conditions whether the increase of cholesterol formation occurs in the liver or is performed outside of the liver, manifesting itself, in the latter case, as a systemic disorder of the reticulum cells and histiocytes

a.) *Increased cholesterol formation in the liver* In the liver, the cholesterol excretion in the bile and the release of newly formed cholesterol into the blood stream seems to be functionally related An increase of cholesterol formation in the liver may therefore involve these functions in such a way that in case more cholesterol is formed and released into the blood stream more cholesterol is excreted into the bile fluid However, the physiological range of the excretory functions of cholesterol in the liver is apparently limited since the concentration of cholesterol in the bile is low and varies but little (16, 49) For this reason increased cholesterol formation in the liver must lead to a considerable accumulation of cholesterol in the blood serum, without primarily involving the other functions of this organ. It is understandable that as long as the liver parenchyma is undamaged the increased amount of cholesterol produced and released into the blood stream will appear as cholesterolester (21)

The clinical syndrome designated by Thannhauser and Magendantz as "primary essential xanthomatosis of the hypercholesteremic type" may be explained on the basis of such a pathogenesis initiated by an increased cholesterol formation in the liver It is manifested by an accumulation of cholesterol in the serum since the physiological facilities for cholesterol excretion in the liver apparently cannot adjust themselves to the increased supply

This syndrome is characterized by 1) xanthoma formation of the skin (xanthoma tuberosa et plana), 2) xanthoma of the tendons whereby nodes are produced consisting of cholesterol-containing foam cells and fibrous tissue, 3) xanthoma of the intima of blood vessels and of the endocardial linings of the heart The incidence of this syndrome in families as recessive hereditary stigma is of great interest In members of the same family the complete syndrome may be present while in others only hypercholesteremia may be found (Incomplete form, forme fruste) (50)

In contrast to other types of hypercholesteremia the serum in this group of cases does not contain increased amount of neutral fat and is therefore not creamy in appearance This significant difference is due to the different mecha

nism resulting in hypercholesteremia It is suggested that in "essential xanthomatosis of the hypercholesteremic type" the hypercholesteremia is caused by an increased formation of cholesterol in the liver not compensated by an increased excretion, while hypercholesteremia associated with an increase of neutral fat (creamy serum) is a phenomenon due to a disturbance of the deposition or transportation of neutral fat as cholesterol accompanies neutral fat wherever it travels and accumulates in the organism In the liver of cases of "essential xanthomatosis of the hypercholesteremic type" anatomical changes may not be detectable in the beginning of the disorder This is understandable, since a functional disturbance of the balance between cholesterol production and cholesterol excretion is assumed to be the pathogenetic factor for this type of essential xanthomatosis If, however, in later stages of the disorder anatomical changes of the liver occur, such changes do not consist of foam cell formation within the liver tissue itself but they appear clinically and anatomically as features of a slowly developing biliary cirrhosis It has been suggested that this special type of biliary cirrhosis be designated "xanthomatous biliary cirrhosis" (50) because its outstanding features are hypercholesteremia with xanthoma formation of the skin of the tendons and of the inner linings of blood vessels In some of these cases also the inner linings of the bile ducts and of the gall bladder are covered with xanthomatous lesions The chemical analysis of the serum in these cases shows an increase of the total cholesterol and also an increase of lecithin to 3–6 times of the normal values The cholesterolesters are prevalent The neutral fat however is normal or only slightly increased The bilirubin is considerably elevated in proportion to the jaundice which has been present for many years In the later stages of the disease, concurrent with the slow progress of the liver damage and the slowly developing cirrhosis, the cholesterol values decrease gradually and the cholesterol cholesterolester ratio changes in favor of the free cholesterol Dehydrocholesterol which is present only in small amounts in normal serum is found to be increased (51)

b ) *Increased cholesterol formation in reticulum cells and histiocytes* Pinkus and Pick (51) were the first to discover that the fat substances in the foam cells are cholesterol and cholesterolesters They advanced the theory that "cholesterol infiltration of certain cells takes place because of an increased cholesterol supply from the blood " There is however a clinically and anatomically well defined group exhibiting xanthoma formation without increased cholesterol supply from the blood presenting normal cholesterol values of the serum Such an occurrence cannot be explained by the assumption of an infiltration of cholesterol due to an increased supply Waldeyer (45) has already suggested that the xanthoma cell is an embryonal cell capable of forming different kinds of lipids, which are retained within the cell and only released by disintegration of these cells Since reticulum cells and histiocytes retain the functional possibilities of embryonal cells it can be assumed that they also are capable of forming various kinds of lipids and also of cholesterol It is conceivable that in these cells a disturbance of the intracellular enzymatic systems concerned with the formation of cholesterol may result in an accumulation of cholesterol within the

cell thereby transforming these cells into xanthoma cells (foam cells) It may be understood that also under these abnormal conditions the cholesterol formed in excess is retained within the cell and not released into the blood stream Such an explanation was applied by Thannhauser and Magendantz (50) to the pathology of a systemic xanthomatous disorder which they designated as "Primary essential xanthomatosis of the normocholesteremic type" singling out the normal cholesterol content of the blood as the leading symptom. As the foam cells in this type occur concurrently with a systemic new growth of granulomatous tissue the disorder was designated by W Chester (53, 54) as "Lipoidgranulomatosis." Recently the pathologists L Lichtenstein and H Jaffe (57) and especially S Farber (55, 56) designated this group as "Eosinophilic granuloma" because eosinophilic cells as well as giant cells are found in the granulomatous tissue beside the nests of foam cells In some juvenile cases nests of foam cells may even be absent or very scarce Whatever designation these authors use they refer to the same disease It is characterized by a special type of a systemic granuloma in which,foam cells singly or in large nests have developed despite the fact that the cholesterol level of the circulating blood is normal In earlier times before all the features of this syndrome were sufficiently studied considerable confusion was caused by the fact that only one or the other organ may be affected by the disease Various names referring to the organ involved are found in the literature such as xanthoma disseminata of the skin, lipid granulomatosis of the bones, Hand-Schuller-Christian syndrome They are all singly or in various combinations in the syndrome (essential xanthomatosis of the normocholesteremic type, eosinophilic granuloma, lipid granuloma) are the skin (disseminata type of xanthoma), the skeleton, dura, brain, lungs, pleura, lymph nodes, liver and spleen (50)

While a description of the clinical picture of this systemic disorder and an elaboration of the differential diagnosis in question has no place in this review, three differential diagnostic points, however, should be stressed in distinguishing the hypercholesteremic from the normocholesteremic type of essential xanthomatosis

1) The skin xanthoma in the hypercholesteremic type are "xanthoma tuberosa et plana" of carotinlike color The skin xanthoma in the normocholesteremic type are "xanthoma disseminata" of sepia or chamois hue Both kinds are also different in their location

2) The organs involved in the "hypercholesteremic type" are the skin, the tendons, the inner layer of blood vessels and possibly the inner layer of the bile ducts This type occurs often in families The organs affected in the "normocholesteremic type" (eosinophilic granuloma) are the skin (disseminata type of xanthoma), the skeleton, dura, brain, lungs, lymph nodes, spleen and liver Tendons and inner layer of the blood vessels are not involved in the "normocholesteremic type" This type of essential xanthomatosis is *not* inherited.

3) If the liver is involved in the "hypercholesteremic" type it differs clinically and anatomically from the liver involvement in the "normocholesteremic"

type (eosinophilic granuloma) In the hypercholesteremic type, jaundice of the obstructive kind is present lasting many years, biliary cirrhosis and in the late stages even symptoms of portal obstruction may develop Foam cells are not formed in the liver tissue In the normocholesteremic type (eosinophilic granuloma) however, in contrast to these findings, jaundice is never present While the liver disease does not develop to biliary cirrhosis, little grayish nodules of granulomatous tissue are found irregularly dispersed in the liver consisting of granulomatous tissue containing nests of foam cells and also eosinophiles

A classification of the various kinds of diseases exhibiting xanthoma formation is suggested by Thannhauser (58)

I Primary Essential Xanthomatosis (Reticular and Histiocytic Cholesterosis
  A Primary essential xanthomatosis of the hypercholesteremic type
    1 Xanthelasma of the eyelids and xanthoma tuberosum et planum
    2 Tendon xanthoma
    3 Xanthoma tuberosum et planum and tendon xanthoma
    4 Xanthoma of the blood vessels and endocardium
    5 Forme fruste of essential xanthomatosis
    6 Xanthomatous biliary cirrhosis
  B Primary essential xanthomatosis of the normocholesteremic type (eosinophilic granuloma)
    1 Xanthoma disseminatum of the skin
    2 Xanthoma disseminatum and diabetes insipidus
    3 Osseous xanthoma
    4 Schüller-Christian syndrome Osseous xanthoma (defects in the membranous bones of the skull), exophthalmus and diabetes insipidus
    5 Generalized xanthoma of the normocholesteremic type (Eosinophilic granuloma) Xanthoma disseminatum, xanthoma of the dura and brain, xanthomatous involvement of the lungs and pleura with consequent pulmonary fibrosis, xanthomatosis of the lymph nodes, hepatosplenomegaly due to scattered nests of xanthoma cells in the spleen and liver

II Secondary Xanthomatosis Due to Hyperlipemia
  A Idiopathic (familial) hyperlipemia with hepatosplenomegaly and secondary xanthoma
  B Secondary xanthomatosis due to diabetic hyperlipemia
  C Hyperlipemia with secondary xanthomatosis in chronic pancreatitis
  D Hyperlipemia in glycogen storage disease
  E Hyperlipemia in lipoid nephrosis

III Localized Xanthoma Formation in Inflammatory Tissue and in True Tumors
  A Xanthoma cells in inflammatory tissue
    1 Inflammatory xanthoma of the breast,
    2 Osteomyelitis, osteitis fibrosa cystica disseminata
    3 Xanthomatous transformation of the mesentery (intestinal lipodystrophy of Whipple)

4 Xantholipomas
B Xanthoma cells in tumors
1 Nevo-xantho-endothelioma
2 Xanthomatous polycystic lymphangiomas
3 Single xanthomatous giant cell tumors
4 Epithelial tumors with xanthoma cells
C Xanthoma cells in other conditions
1 Lipoid proteinosis
2 Necrobiosis lipoidica diabeticorum

Schönheimer and his co-workers (59, 60, 61, 62, 63) have shown that neither herbivorous nor carnivorous animals absorb plant sterols from the intestines Animal cholesterol derived from food or from secretion in the bowels is the only sterol absorbed from the intestines. For this reason a diet low in animal choles terol containing mainly fat of vegetable origin should be given to patients ex hibiting xanthoma with hypercholesteremia (64) In cases of "essential xan thomatosis of the normocholesteremic type" a diet low in animal sterols has no influence on xanthoma formation.

*B Niemann Pick's Disease (Reticular and Histiocytic Sphingomyelinosis)* This disorder was first described by A Niemann in a case reported in 1914 (1) It was L. Pick, in 1927, who differentiated this condition (2) from Gaucher's disease E Klenk (3, 4) discovered that the lipid present in the large pale cells was mainly sphingomyelin These sphingomyelin-loaded cells are found in all organs of the body and are not restricted the lympho-hemopoietic apparatus Comprehensive studies of the disease are included in the monograph of T Baumann, E Klenk, and S Scheidegger, (5) and S J Thannhauser (6) In the pres ence of Niemann Pick's disease sphingomyelin determinations in different organs showed a tremendous increase over the normal However, in the brain the sphin gomyelin content was found in the range of normal values (3, 7, 8, 9) The fatty acids yielded after hydrolysis of sphingomyelin of Niemann Pick organs did not differ from the fatty acids of sphingomyelin prepared from normal organs (10, 11) Sphingomyelin of brain of Niemann Pick disease however was found to contain only stearic acid in contrast to sphingomyelin of normal brain, which yielded lignoceric, palmitic, and stearic acid (12) Other authors (13, 14) have reported that mainly stearic acid is present but also lignoceric and palmitic acid are found in the sphingomyelin isolated from the Niemann Pick brain These studies elaborating the differences of the fatty acids of sphingomyelin cannot be consid ered as final since it was not known at those times that the sphingomyelin isolated by the methods of preparation generally used is mixed with a glycerol containing monophosphatide (15, 16, 17) This substance was shown to be a hydrolecithin containing palmitic acid (18) As the physical properties of hydrolecithin are almost identical with sphingomyelin its presence in sphingomyelin preparations has eluded discovery It is therefore not yet proven that sphingomyelin isolated from Niemann Pick brain may differ from normal sphingomyelin in respect to its fatty acids

L. Pick's conception (2, 19) of the pathogenesis of Niemann Pick's disease was based on the idea that sphingomyelin is taken up from the circulating blood and

deposited and stored in the reticulum cells and histiocytes By this conception it is assumed that the substance deposited and accumulated in a cell is first increased in the serum The sphingomyelin content of the serum of patients suffering of Niemann-Pick's disease however is normal (5, 20) The hypothesis that sphingomyelin is deposited in the cell as a result of its accumulation in the serum is not substantiated by these analytical findings Sperry (21), however, is not of the opinion "that a normal lipid concentration in the serum of patients with lipoidosis rules out the possibility that the lipids deposited in tissues were transported there by the blood, it may indicate that the primary lesion is in the cells, where the deposition takes place and is of such a nature as to cause the uptake of lipids from the serum by a sort of vacuum process " This rather unusual conception of a "vacuum process" is unparalleled in cellular metabolism of animals A deposition and infiltration of reticulum cells and histiocytes with sphingomyelin (22) and with aceton-insoluble lipids obtained from beef brain (23) was only possible by injection of these substances whereby their concentration in the body fluids was abnormally increased

In analogy with the conception of the pathogenesis of essential xanthomosis of the normocholesteremic type (lipid granulomatosis, eosinophilic granuloma) Thannhauser expresses the opinion that also in Niemann-Pick disease an intracellular enzymatic disturbance of sphingomyelin disintegration or new formation leads to an accumulation of sphingomyelin within the cell

Knowledge concerning the enzymes involved in the ana- and katabolism of sphingomyelin is very meager The occurrence of ceramides (fatty acidamids of sphingosin) in organs where also sphingomyelin is found (24) suggests the idea that ceramides are the basic substances from which the synthesis of sphingomyelin proceeds by esterification with phosphorylcholine Vice versa, ceramides may originate from sphingomyelin by splitting of phosphorylcholine from its molecule There is however up to the present no experimental evidence of the enzymes involved in such a reaction so that the pathways used for sphingomyelin formation and disintegration are unknown The methods employed for the estimation of sphingomyelin are described on page 295

The Niemann-Pick cell is pale and foamy in appearance and forty-five or more microns in diameter (25, 26) There is no new growth of granulomatous tissue as is found in essential xanthomatosis of the "normocholesteremic" type (Lipid granulomatosis, eosinophilic granuloma ) Concerning the histiogenesis of the Niemann-Pick cell, opinions vary, since not only the reticulo-endothelial apparatus but more or less all the tissues of the body are involved Bloom (27) expressed the following opinion "In studying the tissues for the source of lipoid-laden phagocytes, certain genetic relationships between some of the various connective tissue cells of the human organism become apparent All stages in the mobilization of the embryonic mesenchymal cells and the mature histiocytes well into large lipoid containing histiocytes can be clearly seen The foam cells develop from the resting endothelial and from the Kupfer cells in the liver, from the reticulum cells of the lymph nodes, the reticulum cells (exclusive of the littoral cells) in the spleen and bone marrow, from the reticulum cells of the medulla of

the connective tissue and the perivascular embryonic cells and phagocytes all over the body "

Chemical analysis of the blood serum in Niemann Pick's disease shows a normal composition of its constituents (5) The neutral fat is slightly increased but as already mentioned, the concentration of sphingomyelin is within normal limits (28) In the blood smear giant reticulum cells probably containing sphingomyelin were found (5) The ganglion cells of the brain are enormously blown up and show disintegration of the tigroid bodies All areas of the brain demonstrate the same degenerative process of the ganglion cells (5) Niemann Pick cells usually do not stain with the customary fat stains, Sudan III or Nile blue sulphate The Lorrain-Smith Dietrich technique is used to demonstrate sphingomyelin in tissue cells.

Niemann Pick's disease has been observed in single cases and also in families. The disease occurs in infants The infants usually are born at full term and with normal weight The onset of the disease is insidious, first there is loss of appetite and corresponding loss of weight until the infant becomes thin and emaciated. The abdomen becomes enlarged. Diarrhea and vomiting result in dehydration Gradually the motor and psychic functions are lost In the advanced phase of the disease the child is apathetic The face has a mongoloid expression, the mouth is open and the tongue protrudes The emaciated extremities have an increased tone and can scarcely move The infant becomes completely debilitated mentally and physically until death terminates its life between the first and second year The skin shows a diffuse brownish yellowish pigmentation There is also pigmentation of the mucous membranes of the mouth and blue-black spots on the tongue and on the gums (so-called mongolian spots) are present The skeletal and nervous system as well as all internal organs are involved in the disease and may produce clinical symptoms (20)

*C Gaucher's Disease (Reticular and Histiocytic Cerebrosidosis)* Under the title "De l'epithelioma primitif de la rate" in 1882, E. Gaucher described a disease which is characterized by an enlargement of the spleen (1) It was not until 1905 that Brill, Mandelbaum and Libman (2) recognized the involvement of the liver and the osseous system as well as the spleen in this disease It was thought that this disease was neoplastic in nature Schlagenhaufer was the first, in 1906, to refer to this disease (3) as a disorder of the lymph hemopoietic system Marchand, 1907, in a study of the histology of the disease (4) pointed out that it was neither neoplastic nor simple hyperplasia but resulted from a deposit of some foreign substance This substance was identified by Lieb in 1927 as cerebroside and was thought to be identical with kerasin (5, 6) Epstein and Lorenz, 1930, referred to Gaucher's disease as analogous to xanthomatosis and Niemann Pick's disease calling attention to the fact that in these three disorders three different types of lipids are found predominantly deposited in large cells of various organs (7) L. Pick (1933) classified these three diseases as disorders of the lipid metabolism suggesting that the different lipids involved in the special type are accumulated in the blood stream and deposited and stored in the reticulum cells (8) A special form of Gaucher's disease occurring only in infancy was described, 1927,

by Oberling and Woringer (9) The infantile form is not restricted to reticulum cells of the lymph-hemopoietic system, but as in Niemann-Pick's disease involves all organs transforming the resting mesenchymal cells as well as the mature histiocytes and reticulum cells into large cells laden with cerebrosides

Since L Pick in his classical description of the lipid disorders (8) advanced the opinion that in Gaucher's disease the lipid accumulation is the result of a metabolic disorder, different theories have been suggested The focal point of Pick's theory is the assumption that cerebrosides are deposited in the cell from the blood stream Such an opinion has as a prerequisite that cerebrosides are present in abnormal amounts in the serum of these patients No one up to the present time, however, has been able to isolate cerebrosides at all from normal serum or from the serum of Gaucher's disease (10, 11, 12) The only reference to the presence of cerebrosides in serum is found in papers dealing with "quantitative" methods of determination of cerebrosides in plasma (13) These methods are indirect methods based on the reduction of sugar liberated by hydrolysis from cerebrosides However Thannhauser and co-workers (13) as well as Brückner (12) were not able to demonstrate measurable amounts of cerebrosides in normal serum Correspondingly in the serum of patients affected with Gaucher's disease not more than traces of a substance giving the Molisch reaction could be detected (10, 11) On the basis of these negative findings it seems justified to assume that cerebrosides are not present in measurable quantities either in the serum of normal individuals or in the serum of patients afflicted with Gaucher's disease A theory explaining the accumulation of cerebrosides in the reticulum cells of Gaucher's disease by an increased transportation and secondary deposition in the cells does not seem therefore to coincide with the actual findings Thannhauser (14) expressed the opinion that cerebrosides are produced within the same cells, in which they are found Consequently a metabolic disorder leading to an overproduction and accumulation of cerebrosides must take place within the cell, where this substance originates For this reason it was suggested that the accumulation of cerebrosides in Gaucher's disease is due to a disturbance of the intracellular enzymes concerned with the ana- and catabolism of cerebrosides, this explanation is analogous to the conception ventured for the etiology of essential xanthomatosis of the "normocholesteremic" type (lipid granulomatosis, eosinophilic granuloma) and for Niemann-Pick's disease, see p 308

In animals injections of cerebrosides as well as injections of cholesterol and sphingomyelin produce an increase of the serum level of these substances above the normal and a secondary deposition in the phagocytic histiocytes (15, 16) Such experiments, however, do not permit the assumption that an analogous sequence of events (accumulation above the normal level in the serum and deposition in the reticulum cells and histiocytes) is responsible for the accumulation of lipids in Gaucher's disease or in essential xanthomatosis of the normocholesteremic type or in Niemann-Pick's disease The lipid cells in these disorders originate while the concentration of the lipids involved in the serum is not higher than that found in normal individuals The formation of lipid-laden histiocytes after injection of various lipids, however, is analogous in its mechanism to the

xanthoma cell formation secondary to hyperlipemia accompanied by hyper cholesteremia See page 297

In normal individuals cerebrosides are found in great quantities in brain and nerve tissue and in very small quantities in spleen (17) and lung (18) It was believed that the cerebrosides accumulated in the organs of patients with Gaucher's disease are identical with kerasin, a galactoside occurring in brain tissue (5, 6) Aghion (19) and especially Halliday, Deuel Tragerman, and Ward (20) however proved conclusively that the cerebrosides found in the organs of Gaucher's diseases are mainly glucosides since their sugar component fermented with yeast This finding later was confirmed by Klenk (21, 22) and other authors (23) The question has been raised whether Gaucher's disease results from the formation of an abnormal, unphysiological glucose-containing cerebroside Klenk, however, demonstrated that the small quantities of cerebrosides present in normal beef spleen yielded both types of cerebrosides, galactosides and glucosides (22) Also in small amounts of cerebrosides isolated from beef lungs both types of cerebrosides were found (24) For this reason it is not permissible to assume that the glucose containing cerebroside found in Gaucher's disease is an abnormal cerebroside The interesting fact, however, remains that in Gaucher's disease mainly a glucose-containing cerebroside is formed and retained within the cells of the involved organs (spleen, liver, lymph nodes, and bones) The presence of a glucose-containing cerebroside as the main constituent of the lipid accumulated in Gaucher organs is an important reason against the assumption of an increased transportation and deposition of cerebrosides as etiology for this disease, since in brain which is not involved in the cases of adult Gaucher's disease the cerebrosides present are galactosides as in normal brain

Very little is known concerning the nature of the enzymes producing cerebroside formation and disintegration A glucoside-splitting enzyme is present in the spleen (25) There is a lack of knowledge concerning the enzymes responsible for the synthesis of cerebrosides Also the intermediary products occurring in formation and disintegration of cerebrosides are not definitely known It was, however, suggested that the ceramides (like lignocerylphingosine) being a constituent of both the cerebrosides as well as the sphingomyelin molecule are the basic compounds parting from which the synthesis of both substances proceeds and vice versa to which their disintegration leads Since lignocerylsphingosine (26) is found to be present in organs together with cerebrosides and sphingomyelin, it was thought possible that sphingomyelin is built from this ceramide by esterification with phosphorylcholine while cerebrosides may result from its combination with a monosaccharide in glucoside linkage This suggestion (27) is expressed as a hypothesis in figure 1

In order to estimate the amount of cerebrosides in organs and serum several methods are employed (28, 29, 30, 31) The principle of these methods is based on the sugar content of the cerebroside molecule The sugar is split from the molecule by acid hydrolysis. The galactose is determined colorimetrically the glucose by fermentation Since the quantities of cerebrosides in normal organs are very small their extraction does not yield pure substances necessary for colorimetric determination.

The Gaucher cells, in contrast to the foam cells in xanthomatosis and in Niemann-Pick's disease, are very large and homogeneous They are usually multinucleated and do not stain dark blue with Lorrain-Smith-Dietrich stain

The adult form of Gaucher's disease occurs in families and singly The onset of the disease is so insidious that the first symptoms may escape observation. An enlarged spleen may develop as the first sign or pain located in the skeletal system may, without visible enlargement of the spleen, constitute the first symptom of the disease Patchy pigmentation of the face similar to that seen in cloasma uterinum may be seen in later stages A wedge-shaped, darkly pigmented thickening of the conjunctiva on both sides of the pupils as well as linear

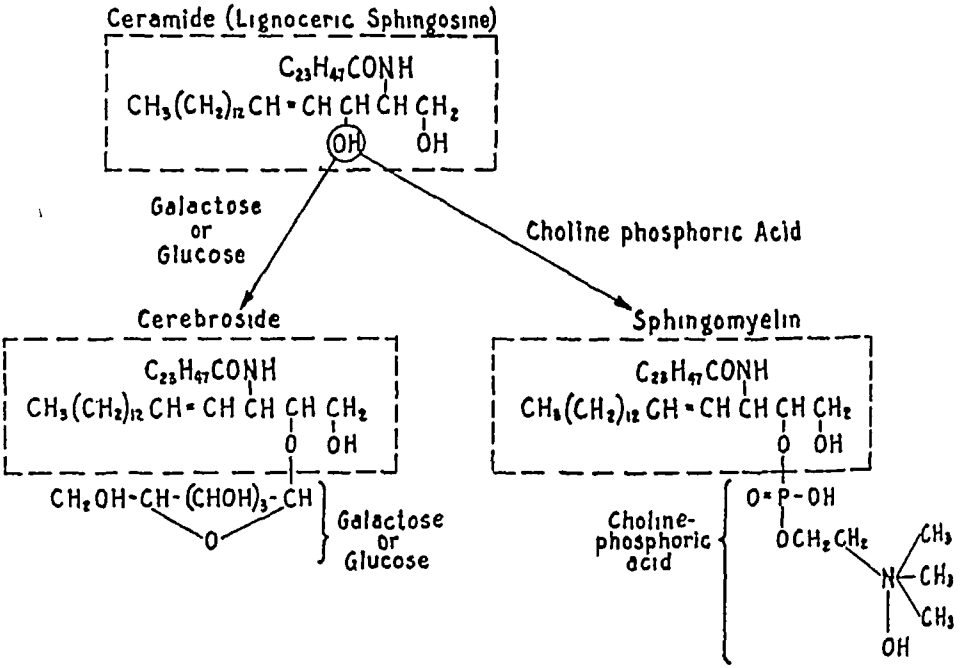



Fig 17

pigmentation of the legs is not a constant symptom of the disease The increase in the size of the liver and the lymph glands is observed subsequent to the enlargement of the spleen The size of the spleen may become enormous and is always more impressive than the size of the enlarged liver Severe pains of the bones occur in intervals and are accompanied by fever In some patients the osseous involvement proceeds without pain and fever and is only discovered by systematic X-ray examination of the entire skeleton The X-ray picture of the femur reveals in most cases a characteristic deformity The waistline of the femur above the condyles disappears because the thinned cortex of the bone seems to be expanded and inflated in this area, probably as the result of the replacement of the marrow by Gaucher cells, giving the distal end of the femur the contours of an Erlenmeyer flask

The diagnosis of Gaucher's disease is made by its clinical features and by the

X-ray pictures of the bones. It should be confirmed by a bone marrow biopsy showing the characteristic Gaucher cell As already mentioned the chemical analysis of the blood serum reveals nothing characteristic The morphological examination of the blood shows in some cases anemia, leukopenia and thrombocytopenia These features of pancytopenia are usually found in cases in which the bone marrow is replaced by other tissues, in the disease under discussion the marrow is replaced by Gaucher cells

The course of the adult form of Gaucher's disease depends on the age as well as on the speed at which the symptoms develop The younger the patient the more rapid is the course of the disease As a rule the course of the disease in adults is protracted and may last a life time Tuberculosis is a frequent complication of Gaucher's disease

In the so-called "infantile form" of Gaucher's disease the development differs, in that not only the organs of the lymph hemopoetic system are involved but Gaucher cells develop in the brain, lungs, and most of the organs (32) The symptoms resulting from the involvement of the brain dominate the clinical picture As a result of the extension of the disease to all organs the clinical features and the rapid progress of the disease are not unlike that observed in Niemann Pick's disease The finding of Gaucher cells in bone marrow biopsies determines the diagnosis.

*D Tay-Sachs Disease (Amaurotic Idiocy, Juvenile Type)* Sachs (1) described in 1896 "A Family Form of Idiocy Generally Fatal Associated with Early Blindness." The ophthalmological features of these diseases had already been published in 1881, by the English ophthalmologist W Tay (2) Since characteristic changes in the retina, characterized by a cherry red spot within a grayish green halo occupying the place of the macula and progressive mental deterioration occur in Niemann Pick's disease as well as in Tay-Sachs' disease it was thought that both diseases are related in respect to their etiology Thannhauser (3), however, showed that the sphingomyelin content of organs was not increased in Tay-Sachs' disease in contrast to the accumulation of sphingomyelin in Niemann Pick's disease Also the histological examination of the retina (4) revealed differences in the histological changes of the retina in both diseases The chemical difference of Tay-Sachs' disease and Niemann's was finally established by E Klenk, by the isolation of a new group of lipids from the brain of infants suffering from Tay-Sachs' disease Klenk named this new group of lipids "gangliosides" (Substance X) The chemical properties of the gangliosides are discussed on page 287 (5, 6) The gangliosides are present in normal brain, but are increased in the brain of Tay-Sachs' disease replacing to some extent the cerebrosides (7)

In Tay-Sachs' disease the ganglioside content of the total solids of the brain is found to be 4–8 per cent, in contrast to 0 3 per cent in the normal brain There is a question whether two types of amaurotic idiocy occur The one type is called Tay-Sachs' disease, the other is designated the "juvenile type of amaurotic idiocy" In the latter type, Klenk found the ganglioside content of the brain only slightly increased (1.5 per cent) Slightly increased values of gangliosides are also found in Niemann Pick's disease It is however definitely established

that Niemann-Pick's disease is entirely distinct from Tay-Sachs' disease as the sphingomyelin content of all organs in Tay-Sachs' disease is normal

*E Pfaundler-Hurler's Disease (Gargoylism)* It is questionable whether changes in lipid metabolism are responsible for this rare syndrome Up to the present there are no chemical findings reported which favor such an etiology of this disease (1, 2, 3, 4, 5)

SUPPLEMENT

*Inositolphosphatides* J Folch (Fed Proc **5,** 134 (1946)) succeeded in further purifying the inositolphosphatide fraction of brain The complete acid hydrolysis of the product yielded inositol phosphoric acid, glycerol and fatty acids in ratios 1 2 1 1 In the intact phosphatide both phosphoric acid groups are esterified with inositol since a short acid hydrolysis resulted in the formation of inositol-m-diphosphoric acid ester

The phosphatide contained 16% inositol It accounts for the total amount of inositol present in the crude cephaline fraction The nitrogen content of 0 6% is far below that required for the presence of one equivalent of base It is not yet established whether the N containing group is a part of the phosphatide molecule or whether it is a contamination

*Cardiolipin* On saponification with alcoholic potassium hydroxide, cardiolipin yields an organic P ester which is very easily hydrolyzed by dilute mineral acids (W C Pangborn, Fed Proc **5,** 149 (1946)) The only hydrolysis products formed are glycerol and glycerophosphoric acid Pangborn assumes, therefore, that cardiolipin represents a phosphatidic acid in which glycerophosphate is replaced by a complex glyceryl-glycerophosphate

*Glycerylphosphorylcholine* E Kahane and J Levy (Compt rend 219, 431 (1944)) found in rat intestines a lecithinase B which hydrolyses lecithin to fatty acids and to a watersoluble choline compound which contained glycerophosphate The properties of the crude fraction obtained by Kahane and Levy are similar to those of the pure glycerylphosphorylcholine isolated from pancreas by Schmidt, Hershman and Thannhauser Kahane and Levy found that their fraction has no pharmacological effects

Independently G Schmidt, L Hecht and S J Thannhauser (J Biol Chem, in print) found that lecithin disappears rapidly during the autolysis of minced rat intestines Simultaneously, free choline and glycerylphosphorylcholine appear in amounts corresponding to those of the hydrolyzed lecithin

E J King and W Aloisi (Biochem J **39,** 470 (1945)) isolated from beef pancreas a watersoluble choline ester of glycerophosphate with the ratios choline glycerophosphate = 2 1

*Determination of phospholipids* G Blix (Biochem Zs **305,** 129 (1940), J Biol Chem **139,** 471 (1941)) determined colamine in hydrolysates of phospholipids by vacuum distillation under 10 mm Hg pressure at 75–80° and following acidimetric titration

*Determination of cerebrosides* Brückner has recently applied the orcinol test to the separate determination of glucose and galactose in mixtures of both sugars

He described on this basis a procedure for the separate determination of galactose and glucose containing cerebrosides (Ztschr physiol Chem 275, 73 1942))

## REFERENCES

### I *Chemistry of the lipins*


(1) Anderson R J Yale J Biol Med 15: 311 1943
(2) Bloor, W R Biochemistry of the fatty acids and their compounds the lipids Reinhold Publishing Corporation New York 1943
(3) Hilditch T P Ann Rev Biochem 11 77 1943
(4) Klenk, E F Ditt and W Diebold Ztschr physiol Chem 232: 54 1935
(5) Klenk, E and J Dittmer Ztschr physiol Chem 244 203 1936
(6) Thannhauser S J and J Benotti Ztschr physiol Chem 253 217 1938
(7) Klenk E Ztschr physiol Chem 242 250 1936
(8) Fränkel, E F Bielschowsky and S J Thannhauser Ztschr physiol Chem 218: 1, 1933
(9) Schoenheimer, R and D Rittenberg J Biol Chem 120: 155 1937
(10) Velick S F J Biol Chem 159: 711, 1945
(11) Cramer, D L and J B Brown J Biol Chem 151: 427 1943
(12) Brode, W R J W Patterson J B Brown and J Frankel Ind and Eng Chem, Anal Ed 16 77 1944
(13) Barnes R B, U Liddel and V Z Williams Ind and Eng Chem Anal Ed 16 1 1943
(14) Francis, F S H Piper and T Malkin Proc Royal Soc London Series A 128: 214, 1931
(15) Velick S F J Biol Chem 154: 497 1944
(16) Ställberg-Stenhagen S and E Stenhagen J Biol Chem 159 255, 1945
(17) Klenk E Ztschr physiol Chem 221 67 1933
(18) Dolby, D E, L C A Nunn and I Smedley MacLean Biochem J 34 1422 1940
(19) Mowry D T W R Brode and J B Brown J Biol Chem 142 679, 1942
(20) Arcus, C L and I Smedley MacLean Biochem J 37: 1 1943
(21) Burr, G O and R H Barnes Ann Rev Biochem 12: 157 1943
(22) Lesuk A and R J Anderson J Biol Chem 139: 457 1941
(23) Thierfelder, H and E Klenk Die Chemie der Cerebroside und Phosphatide J Springer Berlin 1930 page 91
(24) Thannhauser S J and G Schmidt Ann Rev Biochem 12: 238 1943
(25) Sinclair R G and M. Dolan J Biol Chem 142 659 1942
(26) Klenk E and L Clarenz Ztschr physiol Chem 257 268 1938
(27) Klenk E and E Schumann Ztschr physiol Chem 272 177 1942
(28) Spielman M A J Biol Chem 106: 87 1934
(29) Anderson, R J and E Chargaff J Biol Chem 85:.77 1929–1930
(30) Anderson R J J Biol Chem 97 639 1932
(31) Stenhagen, E and S Ställberg J Biol Chem 139: 345, 1941
(32) Sabin F R Physiol Rev 12: 141 1932
(33) Velick S F J Biol Chem 152 533 1944
(34) Ginger L G and R J Anderson J Biol Chem 157 203 1945
(35) Salisbury L F and R. J Anderson J Biol Chem 112: 541 1935–1936
(36) Anderson R. J, R E Reeves M M Creighton and W C Lothrop Am Rev Tubere 48 65 1943
(37) Weitkamp A W J Am Chem Soc 67 447, 1945
(38) Velick S F and J English Jr. J Biol Chem 160: 473 1945
(39) Peck R L and R. J Anderson J Biol Chem 140 89, 1941

(40) LESUK, A AND R J ANDERSON J Biol Chem **136** 603, 1940
(41) FOLCH, J J Biol Chem **146** 31, 1942
(42) LEVENE, P A AND I P ROLF J Biol Chem **72** 587, 1927
(43) PANGBORN, M C J Biol Chem **137** 545, 1941
(44) FOLCH, J J Biol Chem **146** 35, 1942
(45) CHARGAFF, E , M ZIFF AND D RITTENBERG J Biol Chem **144** 343, 1942
(46) ANDERSON, R J J Am Chem Soc **52** 1607, 1930
(47) ANDERSON, R J AND E G ROBERTS J Biol Chem **89** 611, 1930
(48) KLENK, E , AND R SAKAI Ztschr physiol Chem **258** 33, 1939
(49) FOLCH, J AND D W WOOLLEY J Biol Chem **142** 963, 1942
(50) WOOLLEY, D W J Biol Chem **147** 581, 1943
(51) FEULGEN, R , K IMHAUSER AND M BEHRENS Ztschr physiol Chem **180** 161, 1929
(52) BEHRENS, M Ztschr physiol Chem **191** 183, 1930
(53) ANCHEL, M AND H WAELSCH J Biol Chem **145** 605, 1942
(54) FEULGEN, R AND T BERSIN Ztschr physiol Chem **260** 217, 1939
(55) FEULGEN, R AND H GRÜNBERG Ztschr physiol Chem **257** 161, 1939
(56) WAELSCH, H J Biol Chem **140** cxxxvi, 1941
(57) CHIBNALL, A C AND H J CHANNON Biochem J **23** 176, 1929
(58) PANGBORN, M C J Biol Chem **161** 71, 1945
(59) ANDERSON, R J , W C LOTHROP AND M M CREIGHTON J Biol Chem **125** 299, 1938
(60) ANDERSON, R J , R L PECK AND M M CREIGHTON J Biol Chem **136** 211, 1940
(61) GEIGER, W B AND R J ANDERSON J Biol Chem **129** 519, 1939
(62) CREIGHTON, M M , L H CHANG AND R J ANDERSON J Biol Chem **154** 569, 1944
(63) KLENK, E Ztschr physiol Chem **235** 24, 1935
(64) KLENK, E Ztschr physiol Chem **229** 151, 1934
(65) LEVENE, P A AND C J WEST J Biol Chem **16** 549, 1913–1914
(66) LEVENE, P A AND C J WEST J Biol Chem **18** 481, 1914
(67) LAPWORTH, A J Chem Soc **103** 1029, 1913
(68) KLENK, E Ztschr physiol Chem **185** 169, 1929
(69) CARTER, H E , F J GLICK, W P NORRIS AND G E PHILLIPS J Biol Chem **142** 449, 1942
(70) LEVENE, P A J Biol Chem **24** 69, 1916
(71) THANNHAUSER, S J AND E FRÄNKEL Ztschr physiol Chem **203** 183, 1931
(72) FRÄNKEL, E AND F BIELSCHOWSKY Ztschr physiol Chem **213** 58, 1932
(73) TROPP, C AND V WIEDERSHEIM Ztschr physiol Chem **222** 39, 1933
(74) THANNHAUSER, S J AND M REICHEL J Biol Chem **135** 1, 1940
(75) THANNHAUSER, S J AND E FRANKEL Ztschr physiol Chem **203** 183, 1931
(76) BLIX, G Mikrochem Acta **1** 75, 1937
(77) KLENK, E AND F RENNKAMP Ztschr physiol Chem **267** 145, 1941
(78) THANNHAUSER, S J AND J BENOTTI Unpublished experiments
(79) THANNHAUSER, S J AND J BENOTTI Unpublished experiments
(80) NIEMANN, C J Am Chem Soc **63** 3535, 1941
(81) CARTER, H E AND W P NORRIS J Biol Chem **145** 709, 1942
(82) HALLIDAY, N H , H J DEUEL, L J TRAGERMAN AND W E WARD J Biol Chem **132** 171, 1940
(83) DANIELSON, I S , C H HALL AND M R EVERETT Pro Soc Exper Biol and Med **49** 569, 1942
(84) KLENK, E AND F RENNKAMP Ztschr physiol Chem **272** 280, 1942
(85) KLENK, E AND F RENNKAMP Ztschr physiol Chem **273** 253, 1942
(86) WALZ, E Ztschr physiol Chem **166** 210, 1927
(87) BLIX, G Skand Arch Physiol **80** 46, 1938
(88) KLENK, F Ztschr physiol Chem **235** 24, 1935
(89) KLENK, E Ztschr physiol Chem **267** 128, 1940–1941

90) Klenk, E Ztschr physiol Chem. 268 50 1941
91) Chargaff E. Advances in protein chemistry New York 1 1 1944
92) Chargaff E F F Nord and C H Weidenhagen Advances in enzymology and related subjects New York 5 31 1945
93) Cohen, S S and E Chargaff J Biol Chem 140 689 1941
94) Chargaff, F J Biol Chem 142 491 1942
95) Claude, A Science 87 467 1938
96) Stern K G and F Durand Reynals Science 89 609 1939
97) Chargaff E J Biol Chem 161: 389 1945

II *Physiology of the lipins*

(1) Weatherby J H J Cell and Comp Physiol 21 1 1943
(2) Sinclair R. G J Biol Chem 96: 103 1932
(3) Sinclair R G Physiol Rev 14: 351 1934
(4) Sinclair R G J Biol Chem 111 515 1936
(5) Sinclair, R G Biol Symposia 5: 82, 1941
(6) Artom C Arch fisiol 32 57 1933
(7) Artom C Arch intern physiol 36 101 1933
(8) Artom C and G Peretti Arch intern physiol 36: 351 1933
(9) Engel, R W Federation Proc 1: No 1, part II 189 1942
10) Stetten, D and G F Grail. J Biol Chem 144 175 1942
11) Fishman W H and C Artom J Biol Chem 154 109 1944
12) Stetten D, Jr. J Biol Chem 140 143 1941
13) Stetten, D Jr. J Biol Chem 138 437 1941
14) Engel, R. W J Nutrition 24 175 1942
15) Beveridge J M R and C C Lucas J Biol Chem 167: 311 1945
16) MacLachlan P L J Biol Chem 146 45 1942
17) Schachner H B A Fries and I L Chaikoff J Biol Chem 146 95 1942
18) Entenman C, I L Chaikoff and F L Reichert Endocrinology 30: 802 1942
19) Entenman, C, F W Lorenz and I L Chaikoff J Biol Chem 134 495 1940
20) Flock, E V and J L Bollman J Biol Chem 144 571, 1942
21) Lawrence J V and O Riddle Am J Physiol 41 430 1916
22) Laskowski M Biochem Ztschr 278: 284 1934
23) Taurog A, F W Lorenz C Entenman and I L Chaikoff Endocrinology 35 483, 1944
(24) Gortner W A J Biol Chem 159 135, 1945
(25) Boyd, E M Biochem J 29 985, 1935
(26) Hevesy G Ann Rev, Biochem 9: 641, 1940
(27) Chaikoff, I L Physiol Rev 22 291 1942
(28) Fishler M C C Entenman M L Montgomery and I L Chaikoff J Biol Chem 150: 47 1943
(29) Zilversmit, D B C Entenman M C Fishler and I L Chaikoff J Gen Physiol 26: 333, 1943
(30) Reinhardt, W O M C Fishler and I L Chaikoff J Biol Chem 152 70 1944
(31) Hahn, L and G Hevesy Nature 144 204 1939
(32) Patterson J M N B Keevil and E W McHenry J Biol Chem 153: 489 1944
(33) MacFarlane M G and B C J G Knight Biochem J 35 884, 1941
(34) Kyes P Berliner Klin Wchnschr 40 956 1903
(35) Willstätter R and K Lüdecke Ber Chem Ges 37: 3753, 1904
(36) Delezenne C and E Fourneau Bull Soc Chim de France 15 421 1914
(37) Belfanti S Ztschr Immunitätsforschung 44: 347, 1925
(38) Slotta K H and H L Fraenkel-Conrat Ber Deutsche Chem Ges 71: 1070 1938
(39) Fairbairn D J Biol Chem. 157 633 (1945)

(40) SCHMIDT, G , B HERSHMAN AND S J THANNHAUSER J Biol Chem **161** 523, 1945
(41) CONTARDI, A AND A ERCOLI Arch Sc biol **21** 1, 1935
(42) BAER, E AND C S MCARTHUR J Biol Chem **154** 451, 1935
(43) BLOOR, W R J Biol Chem **82** 273, 1929
(44) GORTNER, W A J Biol Chem **159** 97, 1945
(45) FOLCH, J AND D D VAN SLYKE Proc Soc Exper Biol and Med **41** 514, 1939
(46) FOLCH, J , H A SCHNEIDER AND D D VAN SLYKE J Biol Chem **133** xxxiii, 1940
(47) ENTENMAN, C AND I L CHAIKOFF J Biol Chem **160** 377, 1945
(48) ARTOM, C J Biol Chem **157** 595, 1945
(49) WINZLER, R J AND E R MESERVE J Biol Chem **159** 395, 1945
(50) TAUROG, A , C ENTENMAN, B A FRIES AND I L CHAIKOFF J Biol Chem **155** 19, 1944
(51) BLIX, G Mikrochim Act **1** 75, 1937
(52) VIEBÖCK, F AND C BRECHER Ber Deutsche Chem Ges **63** 3207, 1930
(53) VORIS, L , G ELLIS AND L A MAYNARD J Biol Chem **133** 491, 1940
(54) RAMSAY, W N M AND C P STEWART Biochem J **35** 39, 1941
(55) SCHMIDT, G , J BENOTTI AND S J THANNHAUSER J Biol Chem , in print
(56) THANNHAUSER, S J , J BENOTTI AND H REINSTEIN J Biol Chem **129** 709, 1939
(57) BRÜCKNER, J Ztschr physiol Chem **268** 163, 1941
(58) KIRK, E J Biol Chem **123** 613, 1938
(59) ERICKSON, B N , I AVRIN, D M TEAGUE AND H H WILLIAMS J Biol Chem **135** 671, 1940
(60) KAUCHER, M , H GALBRAITH, V BUTTON AND H H WILLIAMS Arch Biochem **3** 203, 1943
(61) WILLIAMS, H H , M KAUCHER, A J RICHARDS, E Z MOYER AND G R SHARPLESS J Biol Chem **160** 227, 1945
(62) PETERS, J P AND E B MAN J Clin Investigation **22** 707, 1943
(63) PETERS, J P AND E B MAN J Clin Investigation **22** 715, 1943

## III *Lipidoses*

(1) WINDAUS, A Ztschr f physiol Chem **65** 110, 1910
(2) SCHÖNHEIMER, R AND W M SPERRY J Biol Chem **106** 745, 1934
(3) PAGE, I H AND W MENSCHICK Biochem Ztschr **221** 6, 1930
(4) SCHÖNHEIMER, R Ztschr f physiol Chem **211** 65, 1923
(5) BERGSTROM, S AND O WINTERSTEINER J Biol Chem **141** 597, 1941
(6) SILBERMANN, H AND S SILBERMANN-MARTYNCEWA J Biol Chem **159** 603, 1945
(7) DELOS, J Compt rend Soc de Biol **136** 787, 1942
(8) SCHÖNHEIMER, R AND F BREUSCH J Biol Chem **103** 439, 1933
(9) HASLEWOOD, G A D Biochem J **33** 709, 1939
(10) DAM, H Biochem Ztschr **232** 269, 1931
(11) PAGE, I H AND W MENSCHICK J Biol Chem **97** 359, 1932
(12) COOK, R P Biochem J **31** 410, 1937
(13) BREUSCH, F L J Biol Chem **124** 151, 1938
(14) BEUMER, H AND H FASOLD Biochem Ztschr **259** 471, 1933
(15) NEKAM, L AND B OTTENSTEIN Klin Wchnschr **14** 641, 1935
(16) THANNHAUSER, S J , E ENDERLEN AND M JENKE Arch f exper Path u Pharmakol **130** 308, 1928
(17) CAMPBELL, J M H Quart J Med **18** 123, 393, 1924-25
(18) SPERRY, W M J Biol Chem **71** 351, 1927
(19) BEUMER, H AND F HEPNER Ztschr f d ges exper Med **64** 787, 1929
(20) HEINLEIN, H Ztschr f d ges exper Med **91** 638, 1933
(21) THANNHAUSER, S J AND H SCHABER Klin Wchnschr **5** 252, 1926
(22) EPSTEIN, E Z AND E B GREENSPAN Arch Int Med **50** 203, 1932 **58** 860, 1936
(23) CANTAROW, A Internat Clin **1** 237, 1935, **1** 272, 1938

(24) Man E B B L Kartin, S H Durlacher and J P Peters J Clin Investigation 24: 623 1945
(25) Schultze W H Verhandl d deutsch path Ges 15 47 1912
(26) Smith M G Bull Johns Hopkins Hosp 36 203 1925
(27) Warren S and H F Root Am J Path 2: 69 1926
(28) Wijnhausen O J Berlin Klin Wchnschr 58 1268 1921
(29) Thannhauser S J Lipidoses Oxford Univ Press p 210 1940
(30) Beumer H and A Löschke München med Wchnschr 80 377 1933
(31) Beumer H München med Wchnschr 84 1007 1937
(32) Thannhauser S J Lipidoses Oxford Univ Press p 223 1940
(33) Holt L E, Jr F X Atlward and H C Timbres Bull Johns Hopkins Hosp 64 279 1939 J Clin Investigation 15 451 1936
(34) Goodman M H Shurman and S Goodman J Pediat 16 596 1940
(35) Thannhauser, S J Lipidoses Oxford Univ Press p 179 1940
(36) Buerger M and Grütz Arch f Dermat u Syph 166 542 1932
(37) Craig L S H Lisser and M H Soley J Clin Endocrinol 4: 12 1944
(38) Thannhauser S J Lipidoses Oxford Univ Press p 251 1940
(39) Gamble J L and K D Blakfan J Biol Chem 42 401 1920
(40) Thannhauser S J and H Schaber Ztschr f physiol Chem 127: 278 1923
(41) Dam H Biochem Ztschr 232 269 1931
(42) Enderlen E S J Thannhauser and M Jenke Klin Wchnschr 5: 2340 1926
(43) Schönheimer R and D Rittenberg J Biol Chem 119: LXXXIII 1937
(44) Bloch K and D Rittenberg J Biol Chem 145 625 1942 159: 45 1945
(45) Waldeyer Virchow s Arch 52: 304 1871
(46) Touton K Vierteljahrschr f Dermat u Syph n F 12: 3 1885
(47) Enderlen E S J Thannhauser and M Jenke Arch f exper Path u Pharmakol 120 16 1927
(48) Thannhauser S J and H Schaber. Klin Wchnschr 5: 252 1926
(49) Schindel L Arch f exper Path u Pharmakol 166 30 1932 168 38 1932
(50) Thannhauser S J and H Magendantz Ann Int Med 11: 1662 1937–38
Thannhauser S J Lipidoses Oxford Univ Press p 61 1940
(51) Schönheimer, R Ztschr f klin Med 123: 749 1933
(52) Pick L and F Pinkus Monatschr f Dermat 5 46 1908
Pinckus F and L Pick. Deutsche med Wchnschr 39: 1426 1908
(53) Chester, W Virchow s Arch 279 561 1930
(54) Chester W and V H Kugel. Arch Path 14 595 1932
(55) Green W T and S Farber J Bone and Joint Surg 40 499 1942
(56) Farber S Am J Path 17: 625 1941 Am J Dis Child 68 350 1944
(57) Lichtenstein L and H L Jaffe Am. J Path 16: 595 1940
(58) Thannhauser S J Lipidoses Oxford Univ Press p 43 1940
(59) Schönheimer R Ztschr f physiol Chem 180: 1 1929
(60) Schönheimer R H Behring and R Hummel. Ztschr f physiol Chem. 192 117 1930
(61) Schönheimer R and L Hrdina Z physiol Chem 212 161 1932
(62) Schönheimer, R H Dam and K v Gottberg J Biol Chem 110: 667, 1935
(63) Schönheimer, R and E A Evans Jr Ann Rev Biochem 6: 139 1937
(64) Thannhauser S J Lipidoses Oxford Univ Press p 110, 1940

## III B

(1) Niemann A Jahrb f Kinderh 79: 1, 1914
(2) Pick L Med Klin. 23: 1483 1927
(3) Klenk E Ztschr f physiol Chem 229: 151, 1934
(4) Klenk E. Ztschr f physiol Chem 235 24, 1935
(5) Baumann T E. Klenk and S Scheidegger. Ergebn d allg Path u path Anat 30: 183 1936

(6) THANNHAUSER, S J Lipidoses Oxford Univ Press p 323, 1940
(7) SOBOTKA, H , E Z EPSTEIN AND L LICHTENSTEIN Arch Path 10 677, 1930
(8) EPSTEIN, E AND K LORENZ Ztschr f physiol Chem 211 217, 1932
(9) THANNHAUSER, S J , J BENOTTI AND H REINSTEIN J Biol Chem 129 709, 1939
(10) KLENK, E Ztschr f physiol Chem 229 151, 1934
(11) KLENK, E Ztschr f physiol Chem 262 128, 1939–1940
(12) KLENK, E Ztschr f physiol Chem 235 24, 1935
(13) TROPP, C AND B ECKHARDT Ztschr f physiol Chem 243 38, 1936
(14) TROPP, C AND B ECKHARDT Ztschr f physiol Chem 245 163, 1936–7
(15) KLENK, E AND F RENNKAMP Ztschr f physiol Chem 267 145, 1941
(16) THANNHAUSER, S J AND J BENOTTI Ann Rev of Biochem 12 238, 1943
(17) SCHUHWIRTH Ztschr f physiol Chem 278 1, 1943
(18) THANNHAUSER, S J AND J BENOTTI Unpublished
(19) PICK, L Am J M Sc 185 601, 1933
(20) THANNHAUSER, S J Lipidoses Oxford Univ Press, p 336, 1940
(21) SPERRY, W M J Mt Sinai Hosp 9 812, 1942
(22) FERRARO, A AND G A JERVIS Arch Path 30 731, 1940
(23) TOMPKINS, E H Bull Johns Hopkins Hosp 70 55, 1942
(24) FRÄNKEL, E , F BIELSCHOWSKY AND S J THANNHAUSER Ztschr f physiol Chem 218 1, 1933
(25) BLOOM, W Arch Path 6 827, 1928
(26) PICK, L Ergebn d inn Med u Kinderh 29 519, 1926
(27) BLOOM, W Arch Path 6 827, 1928
(28) THANNHAUSER, S J , J BENOTTI AND H REINSTEIN J Biol Chem 129 709, 193

III C

(1) GAUCHER, E Thèse de Paris, 1882
(2) BRILL, N E , F S MANDELBAUM AND E LIBMANN Am J M Sc 129 491, 1905
(3) SCHLAGENHAUFER, F Virchow's Arch 187 125, 1906–07
(4) MARCHAND, F München med Wchnschr 54 1102, 1907
(5) LIEB, H Ztschr f physiol Chem 140 305, 1924
(6) LIEB, H Ztschr f physiol Chem 170 60, 1925, 181 208, 1929
(7) EPSTEIN, E AND K LORENZ Ztschr f physiol Chem 192 145, 1930
(8) PICK, L Am J M Sc 185 453, 1933
(9) OBERLING, C AND P WORINGER Rev franç de Pediat 3 475, 1927
(10) THANNHAUSER, S J , J BENOTTI AND H REINSTEIN J Biol Chem 129 709, 1939
(11) DWORACEK, E AND H PESTA Wien Klin Wchnschr 52 332, 1939
(12) BRÜCKNER, J Ztschr f physiol Chem 268 251, 1941
(13) ERICKSON, B N , H J SAUNDERS, M L SHEPHERD, D M TEAGUE AND H H WILLIAMS Proc Soc Exper Biol and Med 45 153, 1940
(14) THANNHAUSER, S J Lipidoses Oxford Univ Press, p 278, 1940
(15) KIMMELSTIEL, P AND E LAAS Beitr z path Anat u z allg Path 93 417, 1934
(16) CHRISTIANSON, O O Arch Path 32 369, 1941
(17) WALZ, E Ztschr f physiol Chem 166 210, 1927
(18) THANNHAUSER, S J AND J BENOTTI Unpublished
(19) AGHION Thèse de Paris, 1934
(20) HALLIDAY, N , H J DEUEL, JR , L J TRAGERMAN AND W E WARD J Biol Chem 132 171, 1940
(21) KLENK, E Ztschr f physiol Chem 267 128, 1940–41
(22) KLENK, E AND RENNKAMP, F Ztschr f physiol Chem 272 280, 1942
(23) DANIELSON, I S , C H HALL AND M R EVERETT Proc Soc Exper Biol and Med 49 569, 1942
(24) THANNHAUSER, S J AND J BENOTTI Unpublished
(25) THANNHAUSER, S J AND M REICHEL J Biol Chem 113 311, 1936

(26) Fränkel, E , F Bielschowsky and S J Thannhauser Ztschr f physiol Chem 218 1 1933
(27) Thannhauser S J Lipidoses Oxford Univ Press p 11 1940
(28) Brand, F C and W M Sperry J Biol Chem 141 545 1941
(29) Brückner, J Ztschr f physiol Chem 268: 103 1941
(30) Brückner, J Ztschr f physiol Chem 275 73 1942
(31) Edmann, P V J Biol Chem 143 219 1942
(32) Abalil, A J and K Kato J Pediat 13 364 1938

III D

(1) Sachs, B J Nerv and Ment Dis 21: 475 1895
(2) Tay W Trans Ophth Soc U Kingdom 1 155 1881
(3) Thannhauser S J Lipidoses Oxford Univ Press p 350 1940
(4) Rintelen F Arch f Augenh 119 332 1925
(5) Klenk, E Ztschr f physiol Chem 262 128 1939–40
(6) Klenk, E Ztschr f physiol Chem 268: 50 1941 270 185 1941
(7) Klenk E Ztschr f physiol Chem. 267: 128 1940

III E

(1) Hurler G and M Pfaundler Ztschr f Kinderh 24: 220 1919
(2) Ellis R W B W Sheldon and N B Capon Quart J Med 5: 119 1936
(3) Harvey R M Am J Roentgenol 48: 732 1942
(4) Lurie L A and S Levy J Am Med Sc 207 184 1944
(5) Larson, L and J A. Lichty Am J Roentgenol 50 61 1943

## ERRATUM

Gerhard Schmidt.

Volume 26, page 275 Lipins and Lipidoses, by S J Thannhauser and

The sentence beginning on line 22 (page 301) should read as follows

They are all a part of the syndrome under discussion The organs which may be involved singly or in various combinations in the syndrome (essential xanthomatosis of the normocholesteremic type, eosinophilic granuloma, lipid granuloma) are the skin (disseminata type of xanthoma), the skeleton, dura, brain, lungs, pleura, lymph nodes, and spleen (50)

PHYSIOLOGICAL REVIEWS
VOL. 20 JULY, 1940 No 3

# CHEMOTAXIS IN LEUKOCYTES

MORTON McCUTCHEON
*Laboratory of Pathology, University of Pennsylvania Medical School Philadelphia*

*Definition* Chemotaxis is a reaction by which the direction of locomotion of cells or organisms is determined by substances in their environment If the direction is toward the stimulating substance, chemotaxis is said to be positive, if away from the stimulating substance, the reaction is negative If the direction of movement is not definitely toward or away from the substance in question, chemotaxis is indifferent or absent

*A Organisms showing chemotaxis* Chemotaxis occurs in a wide variety of both plants and animals (82) For example, the sperm of ferns is attracted by malic acid, a substance present in the egg cell, as was described by Pfeffer (71) in 1884, in the first paper on chemotaxis. Also by chemotaxis, pollen tubes may be attracted to the ovary (65, 48), and the direction of root growth determined Myxomycetes (slime molds) react positively to certain carbohydrates (food) and negatively to adequate concentrations of acids and alkalies (17) Protozoa in general show negative chemotaxis, being repelled by injurious concentrations of various substances (41) In metazoa, directional responses to substances in the environment are often mediated by special sense organs, namely, by the sense of smell, which, for example, directs the male moth toward the female, and crabs toward decayed meat Mammals too may show similar reactions Hence chemotaxis serves in reproduction, feeding, and avoiding harmful substances. Presumably this directional response to chemical stimuli depends on diverse mechanisms in various plants and animals In a field so complex, it seems desirable to limit the present review to the chemotactic behavior of leukocytes.

*B Methods* The first recorded observations on chemotaxis of leukocytes seem to be those of Leber (46), who, in 1888, reported results of experiments on rabbit's cornea. Following irritation, leukocytes were seen to move from all directions into the avascular cornea, following straight lines toward the irritated area.

*Capillary tube method* In addition, Leber introduced short lengths of capillary tubes into the anterior chamber of the rabbit eye The tubes contained substances to be tested for chemotactic effect, such as bacteria, which appeared to attract many leukocytes. The capillary tube method enjoyed considerable popularity for some years. A great variety of substances, liquid or solid, were placed in the tubes, which were inserted into the peritoneal cavity (57), anterior chamber of the eye, or subcutaneously (5), or into exudate removed from the body (79) Subsequently the tubes were withdrawn, the contents expressed, and the numbers of leukocytes attracted by different substances were compared By this method was obtained much information of doubtful validity, for, as demonstrated by Pfoehl (72) and Ruchlädew (76), the method is subject to grave errors Into the tubes leukocytes enter not only by amoeboid motion, but by floating as well, as

the result of convection currents These are set up by differences between the liquid in the tubes and that in the tissues outside, differences for example, in osmotic pressure Under these conditions, erythrocytes as well as leukocytes may be found in the tubes, and since erythrocytes obviously have been carried in passively, the same may be true of leukocytes, therefore their presence is not valid evidence of chemotactic attraction

*Micro-injection method* The uncertainties and errors of the capillary tube technic made a more reliable method desirable, one that should prevent the leukocytes from floating and permit one to make a record of their movements The experiments of Clark and Clark (10, 11) met these requirements They injected the material to be tested into the transparent tail of the anesthetized tadpole With the microscope, leukocytes were seen to emigrate from the blood vessels and then to advance by amoeboid motion toward attracting substances, such as starch grains or a droplet of croton oil Camera lucida drawings gave conclusive evidence that leukocytes were attracted by several of these test objects For the *in vivo* method, any transparent tissue, such as mesentery or tissue in the rabbit ear chamber is suitable Nevertheless the method has been little employed for the study of chemotaxis, perhaps because of technical difficulties

While speaking of *in vivo* experiments, it should be emphasized that it does not suffice to inject a test material into living tissues, later to sacrifice the animal and to examine the tissues by histological methods Any even slightly irritating substance thus injected excites emigration of leukocytes, as does, indeed, the injection itself, unless carried out with the utmost precaution, as was done by Clark and Clark Thus emigration of leukocytes is not valid evidence of a chemotactic response (see below, under Relation of Chemotaxis to Emigration) Rather one must observe with the microscope the direction taken by the emigrated cells, and make sure that they move in more or less straight lines toward the test substance, before one is justified in concluding that the reaction is positive chemotaxis

*Slide-coverslip method* Even before the experiments of Clark and Clark, a simple method *in vitro* was devised by Comandon (21) that met the requirements of direct observation of leukocytes and recording their paths He allowed a drop of blood to spread between slide and coverslip, and observed with the microscope and recorded in motion pictures the direction of leukocytes relative to the test object Thus he observed the approach of avian leukocytes to erythrocytes infected with malarial organisms Also he was able to contrast the positive response to starch grains with the lack of chemotaxis to carbon particles (22)

The slide-coverslip method is suitable for testing the chemotactic effect of solid particles, such as bacteria, carbon, etc , and of tissue fragments Liquids may be tested if they are adsorbable on particles such as carbon, titanium dioxide or kaolin The method has the great advantage that individual leukocytes may be observed with the microscope, and their paths of migration relative to the test object may be recorded with the aid of a camera lucida or similar device, as they move toward or away from the test object One may ascertain whether the cell is advancing by amoeboid motion rather than being carried by convection

currents—(floating is impossible when the suspending medium is blood, or plasma not too much diluted)

Semiquantitative studies of chemotaxis were made by McCutcheon, Wartman and Dixon (55, 23), with the simple slide-coverslip technic Either whole blood was used, or a suspension of leukocytes in plasma, permitting 10 to 20 cells to be observed in one microscopic field Suspensions of leukocytes were obtained with the method of deHaan (37), by injecting physiological salt solution into the peritoneal cavity of rabbits and removed the resulting exudate several hours later The leukocytes, nearly all granulocytes, were then resuspended in plasma, a drop of which was used in making the preparation (52)

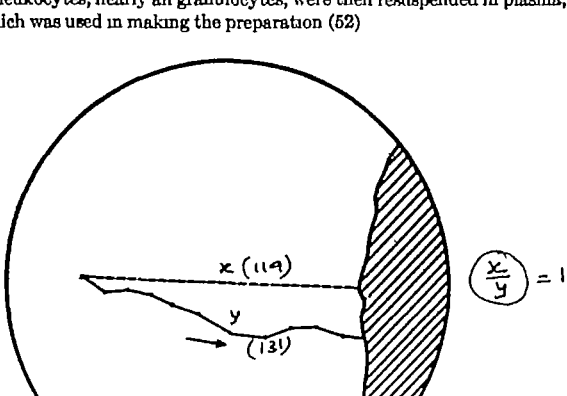



Fig 1 Method for computing the value of chemotaxis

From a graphic record (fig 1) obtained with a drawing ocular or camera lucida, the value of chemotaxis may be obtained for each leukocyte by finding the ratio of net approach to total path travelled in the same time Thus the leukocyte in question was 119 microns from its target at the first observation and 0 micron at the last, hence the net approach was +119 microns The total path for this time was 131 microns The ratio is +119/131 = +0 9, which is the chemotactic ratio The limiting values for this ratio are +1 0, if the leukocyte should move directly toward the object, and −1 0 if it moves directly away (23)

If the simplifying assumption is made that 2 lots of leukocytes are moving at the same average speed, their chemotactic responses may be compared merely by comparing their average net approaches For example, the leukocyte represented in figure 1 approached its object at 119/11 = 10 8 microns per minute (15)

*The tissue culture method* A useful method was worked out by Meier (60), who adapted the plasma hanging drop, as used in tissue culture, to the study of of chemotaxis Blood is centrifuged and the buffy coat, i e , the leukocytes, are removed in a layer and introduced into a hanging drop of plasma, which then coagulates Lymphocytes may be obtained from explants of lymph nodes, macrophages from explants of omentum or other organs The test object, such as a clump of bacteria, is placed at a known distance from the leukocytes, the reactions of which are then observed with the microscope If the test substance is in liquid form, it is drawn into a capillary tube, one end of which is sealed, while the open end is placed in proximity to the leukocytes Leukocytes then migrate from all sides of the explant, but if attracted by the test object, the migration zone is wider on the side of the test object than on the opposite side The reverse is of course true if leukocytes are repelled

The resulting distribution of leukocytes may be photographed after one or more hours By measuring the migration zones with a planimeter, Coman (18) was able to compare the reactions of different kinds of leukocytes to different test objects

Other methods have generally been less dependable An *in vitro* method was developed by Jochims (42), who allowed test substances to diffuse through a collodion membrane into a chamber containing leukocytes in liquid suspension With this apparatus, he reported highly sensitive reactions of leukocytes to differences in H ion concentration This would be of great interest if one could be sure that the leukocytes were not floating, instead of progressing by amoeboid motion That is always a danger in liquid medium, unless the cell is under constant microscopic observation

*C The behavior of leukocytes showing chemotaxis* may be studied in the slide-coverslip preparation described above, using either whole blood or exudative leukocytes For establishing a basis for comparison, the behavior of leukocytes will be described when no chemotactic substance is present When the preparation is observed with the high power of the microscope the polymorphonuclear leukocytes are seen to spread on the glass and to display amoeboid motion Locomotion begins almost at once In the absence of a chemotactic substance, the leukocytes pursue paths that change direction from one minute to another, that is, they move at random The rate of locomotion varies with the temperature (51) and with unknown factors affecting individual cells, under good conditions, human polymorphonuclear leukocytes at 37°C were found to move at an average rate of about 34 microns per minute (50)

To demonstrate chemotaxis in such a preparation, a minute clump of bacteria, such as staphylococcus, is placed on the glass slide and allowed to dry Then a drop of blood is superimposed and made to spread between slide and coverslip When the polymorphonuclear leukocytes in the same microscopic field as the bacteria are observed, practically all are seen to move toward the bacteria, not in a straight line but in a path that deviates a little to one side or the other, often becoming more nearly straight as the leukocyte approaches the bacteria (55) Reaching the bacteria, the cells almost or quite cease moving as they begin phagocytizing

*The chemotactic field. its width* If microscopic fields are examined at increasing distances from the bacteria, the percentage of leukocytes moving toward the bacteria becomes less. Thus, of leukocytes distant 0 4 mm or less from the bacteria, 89 per cent moved toward the bacteria in a given minute, while of leukocytes 0.5 to 0.8 mm distant, only 70 per cent did so Further away a field was reached in which only approximately 50 per cent of cells moved toward the bacteria, that is, motion was here at random In the set up described, the width of the zone of attraction was about 1 mm (54) Compared with this distance Comandon (22) found a zone of attraction about a starch grain of at least 0.2 mm

*The chemotactic field its time relations* There are two other points of interest about chemotaxis under the conditions described First chemotaxis is usually of maximal intensity as soon as the leukocytes begin to move, within a minute or two, that is, almost all the cells close to the bacteria move toward them in nearly straight lines. Little diminution in chemotaxis may be noted for 2 hours or more. Second, the width of the zone of attraction does not increase with time but remains about the same (54) These two observations are consistent with the supposition that an attracting substance is washed off the bacteria when the preparation is made, and is spread to an effective distance of about 1 mm , and that the substance is non-diffusible However, the chemotactic effect of bacteria or other particles cannot be diminished by repeated washings in distilled water Presumably the merest traces of the attracting substance are enough to elicit maximal chemotactic response

*The rate of locomotion is not affected by chemotaxis*, thus, leukocytes move no more rapidly when responding to chemotactic stimuli than when moving at random (25) For example, in one series of experiments, leukocytes moving toward *Staphylococcus albus* travelled at the rate of 28 ± 7 6 microns per minute, whereas leukocytes too far away from the bacteria to show any chemotactic response moved at least as fast, 32 ± 7 4 microns per minute. Thus chemotaxis does not affect the rate of amoeboid motion, rather is chemotaxis a directional response superimposed on amoeboid motion

*D The kind of leukocyte showing chemotaxis* is chiefly the polymorphonuclear leukocyte (granulocyte), and this cell has been observed to give the response in mammals, birds (21) and amphibia (14) In man, the neutrophile is the type of granulocyte studied most, though eosinophiles also show chemotaxis

Ingraham and Wartman (39) studied chemotaxis in *eosinophiles* of a man who had leukemia, and also observed eosinophiles from bullae of a patient with dermatitis herpetiformis. Blood eosinophiles from the leukemic patient, observed by the slide-coverslip method, moved toward *Staph. aureus* at the rate of 9.0 microns per minute, this rate being not significantly different from that of neutrophiles of a normal man (7.5 microns per minute) Eosinophiles from bullae of the patient with dermatitis herepetiformis showed somewhat less response, probably because these leukocytes had degenerated As eosinophilia is often excited by animal parasites, these authors observed the reaction *in vitro* of these cells to *Trichinella spiralis* The organism was dried and ground, and

used as a test object with the slide-coverslip technic This material attracted eosinophiles from the leukemic patient no more strongly than it attracted neutrophiles from a normal person The values were respectively 5 8 and 7 0 microns per minute, and the difference was not significant Rosegger (75), too observed chemotactic responses in eosinophiles

*Lymphocytes* do not show chemotaxis to any substance yet tested (14) The difference between lymphocytes and polymorphonuclear leukocytes in this respect was evident where these two types of cells were observed in the same field of the microscope (23) In slide-coverslip preparations polymorphonuclears were attracted by various bacteria, but lymphocytes moved at random, toward or away from bacteria, often changing direction Thus toward *Staph albus*, the chemotactic ratio of polymorphonuclears was +0 65, of lymphocytes, 0 00 In the presence of tubercle bacilli values were respectively +0 58 and −0 10 (the latter not significantly different from 0 00), nor were lymphocytes attracted by caseous material from tuberculous lungs (24)

Similar results were obtained by Coman (18) in tissue culture by planting staphylococci, tubercle bacilli or clumps of aluminum silicate in plasma, a millimeter or two from a fragment of rat lymph node As lymphocytes migrated into the plasma, their direction of locomotion was not affected by the bacteria or silicate, they formed a migration zone as broad on the side facing these test objects as on the opposite side Under these same conditions, polymorphonuclears were attracted by the bacteria and repelled by aluminum silicate

In the tissues, lymphocytes migrate from blood vessels much as do polymorphonuclears (12), and cells generally regarded as lymphocytes accumulate about tubercles, malignant tumors, transplants, and in chronically inflamed tissues Such accumulations of lymphocytes suggest that they may have been attracted chemotactically Though this may be true, other explanations are possible lymphocytes may have wandered into position through random movements, but, once there, may be prevented from leaving, they may have multiplied locally, or cells resembling lymphocytes may actually be degenerated polymorphonuclears (14)

*Chemotaxis in monocytes* Since these cells are the most efficient of all mammalian cells as phagocytes, it might be confidently expected that they would show positive chemotaxis Observations in fixed and sectioned tissues support this expectation For example, when tubercle bacilli are injected in animals, monocytes accumulate about them as if attracted chemotactically Similarly in pneumococcic pneumonia of man and experimental animals, monocytes regularly move into the pulmonary alveoli in great numbers shortly before recovery, and there apparently play a decisive part in terminating the infection (Robertson, 74) Further, direct observation of living amphibian tissues has given evidence that monocytes react chemotactically (14)

It is astonishing, therefore, that *in vitro*, mammalian monocytes appear to show only weak chemotaxis or none at all When bits of rat omentum were explanted into plasma, and clumps of bacteria were placed nearby, monocytes migrated promptly from all sides of the explant, but failed to show chemotaxis, as the

migration zone was of equal thickness on all sides (Coman, 18) Under the same conditions, polymorphonuclears showed an asymmetrical migration zone, which was broader on the side toward the bacteria, giving evidence of positive chemotaxis. When the test object was aluminum silicate, the migration zone of monocytes was narrower on the side facing the test object than on the opposite side, giving evidence of negative chemotaxis, but the effect was only slight, while statistically valid, there was actually only slight repulsion, in contrast to strong negative chemotaxis shown by granulocytes under the same conditions

Recent experiments by Jacoby (40) suggest that monocytes may in fact show positive chemotaxis *in vitro*, but that the attracting influence extends only a short distance When monocytes were planted in serum and adhered to the glass bottom of a Carrel flask, a cell here and there showed death changes. Photographs of the culture showed that neighboring monocytes, to a distance of about 25 microns, converged from all sides on the degenerated cell, this response ending in phagocytosis of the dead cell

The question as regards chemotaxis in monocytes is not completely answered From present evidence it seems likely that chemotaxis plays a smaller part in the activity of these cells than it does in polymorphonuclear leukocytes

*E Substances that excite chemotaxis* As chemotaxis is a directional reaction to substances in the environment, it is now appropriate to inquire what substances or particles excite this reaction

I *Microorganisms* Bacteria are the most important source of attraction, as chemotaxis to bacterial products aids in combatting infection Apparently bacteria in general excite positive chemotaxis, though under conditions to be mentioned presently, the reaction may be inhibited or masked When tested by the slide-coverslip method, human and rabbit granulocytes were attracted by a variety of bacteria including pyogenic cocci such as staphylococci, streptococci, pneumococci, as well as by such diverse microorganisms as typhoid, tubercle, colon and Welch bacilli (53) An exception was noted in certain strains of beta hemolytic streptococci, which either failed to excite chemotaxis, or else possibly repelled the leukocytes (52) Heat killed bacteria attracted leukocytes as well as did living ones (33) In these experiments, leukocytes were observed while in close proximity to bacteria If, however, there was a greater distance, one or two millimeters, between leukocytes and bacteria, leukocytes were not attracted by some bacteria, such as M tuberculosis, but were attracted by Esch. coli, staphylococci, pneumococci and streptococci (60) Thus the distance over which attraction occurs depends on the particular microorganism, perhaps being greater in the case of rapidly growing bacteria than in the slowly growing acid-fast organisms. Dead bacteria at a distance of 1 or 2 millimeters from leukocytes excited no chemotactic response

*In vivo*, bacteria in general give off chemotactic substances, but the effect of these is not infrequently masked by other and poisonous bacterial products, which retard or stop locomotion of leukocytes, so that the cells are unable to reach the bacteria Consequently the number of granulocytes that collect about bacteria in the tissues varies widely Moreover some bacteria, such as the

pyogenic cocci, attract leukocytes for as long a time as the bacteria grow—in staphylococcic osteomyelitis this may be for years—whereas more slowly growing organisms such as *M tuberculosis* attract granulocytes only at certain times. Thus when tubercle bacilli were injected into the blood stream of animals, granulocytes collected where the bacilli lodged, and this happened within 24 hrs Afterwards these cells were replaced by monocytes But again after a week or two when the tubercle becomes necrotic, a second wave of granulocytes surrounded the bacteria (Lurie, 49, Woodruff, 87)

*Toxic bacterial products and "negative chemotaxis"* Reference has been made to the crippling effects on leukocytes of substances produced by many bacteria In typhoid fever granulocytes are generally absent from the lesions, this is probably the result of poisoning of the bone marrow, so that maturation of granulocytes is prevented and the number of these cells in the blood is reduced. In *Clostridium Welchii* (gas bacillus) infections, leukocytes are paralyzed by a soluble toxin produced in the lesion, consequently leukocytes are unable to approach the bacteria (29) Similar effects may be produced by virulent strains of staphylococci (Stevenson and Reed, 81) streptococci and other microorganisms.

The fact that virulent bacteria may inhibit reactions of leukocytes has important consequences Such infections, unchecked by phagocytic cells, tend to spread rapidly and to destroy life Indeed virulence may signify chiefly that certain strains of bacteria produce substances that inhibit the activity of leukocytes Inhibition of leukocytic movement may, however, be overcome by the presence of antibodies (70), or may be prevented by previous immunization of the animal (57) In these ways, toxic bacterial products are neutralized.

Similar effects of toxin-producing bacteria have been noted *in vitro* An early investigator of chemotaxis, Massart (57), inserted capillary tubes intraperitoneally, if these tubes contained virulent microorganisms, few leukocytes collected in them, in contrast to great numbers of cells in tubes containing non-virulent bacteria Recently, the effect of different strains of staphylococcus was tested on the migration of cells from explants of mouse spleen In the presence of virulent strains, the migration zone was narrower than when less virulent strains were tested (81) Morgan and Upham (67) found that typhoid bacilli retarded migration of cells from an explant of guinea-pig spleen, as compared with the effect of *Staph albus*, while a protein-free antigen of *E typhosa* was toxic enough to inhibit migration

Such interference with the motility of leukocytes, preventing them from approaching and phagocytizing bacteria, has usually been attributed to negative chemotaxis This is probably incorrect, and is due to misunderstanding of the term "negative chemotaxis" As defined above, negative chemotaxis is the reaction of moving away from a substance in the environment If leukocytes showed negative chemotaxis to certain bacteria, one might expect to be able to demonstrate this reaction *in vitro*, but thus far no definite negative chemotaxis to bacteria has been so demonstrated It seems more likely that bacterial poisons injure leukocytes, impairing their power of locomotion Excessive numbers of bacteria in a culture of leukocytes may soon cause migration to cease

(King, Green and Henschel, 43) Bacterial substances that kill leukocytes have been called leukocidins.

*How do bacteria produce their chemotactic effects?* There are two possibilities first, bacteria may produce substances in the course of metabolism that attract leukocytes, second, bacteria may injure tissues, which then give off attracting substances *In vivo* it is difficult or impossible to separate these two processes, and probably both occur at the same time *In vitro*, however, tissue damage may be entirely avoided, so that under these conditions, chemotaxis is a response to bacteria directly (26) Even so, under these experimental conditions *there is another possibility, that bacteria may attract leukocytes* by means of substances, such as polypeptides, absorbed from the culture medium That this is not necessarily true was shown by Meier (61), who used no artificial culture medium but grew bacteria in plasma, then freed the plasma of bacteria by filtration, and showed that the plasma had acquired the property of attracting leukocytes.

As to the nature of this metabolic product, it is probably a protein or a heat stable (Grand and Chambers, 35) protein derivative That bacterial proteins are chemotactic has long been known (7) The molecular size of the attracting substance, as indicated by its filterability, is of the same order as proteins (61), and its lack of diffusibility, mentioned earlier, is consistent with its being protein. From staphylococci, Menkin (64) obtained an attracting substance having the properties of polypeptides, which are protein derivatives.

Several fractions of the tubercle bacillus were studied by Wartman and Ingraham (83, 84) for their chemotactic properties Tubercle protein, when tested in its undissolved form, failed to attract leukocytes, but when dissolved and adsorbed on charcoal, excited positive chemotaxis, the index being +0 69 Tubercle phosphatide was toxic to leukocytes but when adsorbed on titanium dioxide elicited weakly positive chemotaxis (index +0 43) A polysaccharide from tubercle bacilli was found to induce negative chemotaxis in leukocytes of both man and rabbit In each case the index was −0.34

Somewhat similar results were obtained with fractions of hemolytic streptococci (27) Little or no attraction was produced by a carbohydrate fraction adsorbed on kaolin (index +0 18) nor by a protein free, non antigenic hemolysin (index −0 03) An antigenic protein-carbohydrate complex elicited positive chemotaxis (index +0 64), but this result was not confirmed by later (unpublished) experiments, so the effect of this fraction remains in doubt

*Viruses* There is no direct evidence as to whether or not viruses attract leukocytes, but it is inferred that they do not, as granulocytes are usually few or entirely absent in tissues infected by viruses, provided there is no associated infection by bacteria The intracellular location of viruses during multiplication may account in part for their failure to attract granulocytes

*Protozoa.* The organism of avian malaria was observed by Comandon (21) to excite strong positive chemotaxis. Leukocytes moved directly toward parasitized red cells and pushed them along until they burst. The leukocyte might then move toward the nucleus of the red cell, but more often it followed the

parasite and ingested it along with grains of hematin The leukocytes were said to move as fast as 1 to 2 microns per second, and seemed to travel more rapidly as they neared the parasitized cell, though no measurements were given to support this statement

*Yeasts* *Torula histolytica* causes little inflammation and attracts few leukocytes in tissues *In vitro*, leukocytes were attracted at least for a short time (53)

II Chemotaxis excited by *products of injured tissue* It is well known that exudation of leukocytes into tissues may be caused by a variety of injuries, even in the absence of infection Thus large numbers of leukocytes may be found in sterile blebs resulting from burns (Moon 66), as well as in irradiated tissues (9), and may be attracted by injecting various intensely irritating substances such as turpentine These effects are generally believed to be due to chemotaxis, an explanation supported by experiments *in vitro* Thus leukocytes were accumulated in capillary tubes containing products of tissue injury (58), they were attracted by dried leukocytes (55) and by a thermolabile substance released by injured muscle fibers (35) Silverman (80) investigated attracting substances in injured human skin, and found that pulped skin excited strong positive chemotaxis (index +0 83) The attracting substance was soluble in water After extraction with water, pulped skin no longer attracted leukocytes (index +0 05), but from the aqueous extract the attracting substance might be recovered by adsorption on kaolin (index +0 81) This substance was thermostable and gave reactions for protein

*Histamine* Among products of injury, histamine was early thought of as a substance possibly attracting leukocytes, because several of the processes found in inflammation may be produced by injection of histamine

Wolf (86) made chambers with agar impregnated with histamine, filled the chamber with 2 drops of blood, and subsequently counted the number of leukocytes adhering to the agar She reported a strongly positive chemotactic effect as compared with control chambers Others have failed to confirm this effect of histamine Bloom (6) found no attraction of leukocytes in capillary tubes containing histamine Grant and Wood (36) injected histamine into various tissues, but failed to obtain a leukocytic response Moon (66) implanted pith, impregnated with histamine, into the peritoneal cavity, but no more leukocytes were attracted than when the pith contained only serum After extracting the chemotactic factor from skin pulp, Silverman (80) impregnated the pulp with histamine With the slide-coverslip method, the chemotactic ratio was +0 25 Histamine adsorbed on kaolin yielded a ratio of −0 13 Silverman concluded that there was little if any chemotactic response

In contrast to the predominatingly negative results *in vitro*, histamine may cause abundant emigration of leukocytes from blood vessels This result was observed by Abell (1), who introduced histamine into the moat of the rabbit ear chamber It is possible however, that the emigration of leukocytes was not a chemotactic reaction, but rather a reaction to histamine-induced changes in vascular endothelium This possibility is discussed below

*Leukotaxine* Menkin (62) produced sterile exudates in dogs and obtained by fractionation a thermostable, dialyzable substance having the properties of polypeptide. This substance, which he called leukotaxine, attracted leukocytes both in tissues and *in vitro* A substance with similar physiological properties was obtained by tryptic digestion of serum proteins (63) This work was confirmed by Duthie and Chain (28), who obtained by digestion of fibrin a polypeptide that excited positive chemotaxis

Thus injured tissues are an important source of chemotactic material, and the reaction is advantageous, eliminating damaged tissue through phagocytosis and digestion by leukocytic enzymes

III *Carbohydrates* Grains of starch attract leukocytes both in tissue and *in vitro* When starch grains were injected into the transparent tail of the tadpole, leukocytes emigrated and moved toward the grains, which were then phagocytized and digested (11) Chambers and Grand (8) placed starch grains in capillary tubes and observed that leukocytes from the buffy coat of fowl's blood and from the spleens of chick embryos and mice were attracted to and entered the tubes, clustering in masses about the starch grains until these were digested. Comandon (22) too, observed positive chemotaxis to starch. Also other polysaccharides excited positive chemotaxis, notably glycogen (8) and agar (14, 8) Threads of raw cotton were found to attract leukocytes to the cut end, but not to other parts of the thread, as if attracting substance escaped from the cut end Silk threads, on the contrary, were not chemotactic (8) Chambers and Grand regarded attraction by polysaccharides as due to products of serum amylase digestion, and in support of this explanation reported that polysaccharides were not chemotactic in the absence of serum, and also that various sugars attracted leukocytes. Sheldon (78), using the method of Chambers and Grand confirmed attraction by starch and glycogen but not by sugars Chemotaxis to carbohydrates appears to be a feeding reaction just as is slime molds, which are attracted by various carbohydrates (17)

IV *Substances exciting negative chemotaxis* in leukocytes Considerable confusion exists in the literature as to the use of the term negative chemotaxis, as many workers mean by it absence of chemotaxis, while others mean a repelling effect, the opposite of positive chemotaxis The term should be used only in the latter sense, to signify a reaction by which the leukocyte moves away from a substance, that is, moves from higher to lower concentrations.

The possible negative chemotactic effect of certain bacteria was mentioned in a previous paragraph, where it was stated that no clear cut example of negative chemotaxis to bacteria has yet been recorded, though Wartman and Ingraham (84) reported negative chemotaxis to tubercle carbohydrate

A preparation of aluminum silicate (marketed as Lloyd's reagent) induced negative chemotaxis in human and rabbit leukocytes (52) In slide-coverslip preparations, practically all the leukocytes in the field of the microscope were seen to move away from the particles in almost straight lines Under these conditions, exudative rabbit cells moved away at the average rate of about 7 microns per minute the reaction being about as strong as, but in the opposite

direction to, positive chemotaxis to bacteria under similar conditions (20) The significance of this repelling effect of silicate (not shared by silica) is not understood It is astonishing that the only substance thus far shown to cause strong negative chemotaxis in leukocytes is one which the cells would not be likely to encounter naturally Myxomycetes also are repelled by Lloyd's reagent (17) The subject of negative chemotaxis in leukocytes needs further investigation

V *Chemotactic effects of particles* having low solubility in water The repelling effect of aluminum silicate has just been noted Collodion particles have the opposite effect, strongly attracting leukocytes (25) Both these substances have low solubility in water, and their effects on leukocytes are not lessened by repeated washing in distilled water It is remarkable that particles having these properties should excite any reaction at a distance In the case of collodion, these particles are known to adsorb proteins readily, and it may be they had become contaminated with minute amounts.

Most other fairly insoluble particles—silica (54), carbon (15), titanium dioxide (84),—exert no chemotactic effect under the best controlled conditions Hence chemotaxis to these particles is absent Manganese dioxide was reported by Fenn (30) to excite positive chemotaxis In slide-coverslip preparations, he observed that leukocytes moved toward the $MnO_2$ particles, although they showed no reaction to quartz particles which they passed on the way Quartz and carbon particles were encountered only by chance Chambers and Grand (8), on the contrary, found no attraction to be exerted by $MnO_2$

VI *Miscellaneous substances* Chambers and Grand (8) found that physiological solutions, hyper- and hypotonic solutions, and solutions of various salts did not excite chemotaxis Olive oil, oleic and stearic acids, sodium and potassium palmitate were indifferent substances As several of the substances mentioned are strongly surface active, these investigators dissented from the view of Schade (77) and of Häbler and Weber (38) that chemotaxis depends on the surface activity of the exciting substance

*Hydrogen and hydroxyl ions* Jochims (42) reported that solutions between pH 5,8 and 6,3 attracted leukocytes, between 4 4 and 4 9 were negatively chemotactic, and from 6 9 to 8 5 were indifferent These results cannot be unreservedly accepted without confirmation, as Jochims used liquid medium in which leukocytes might have been transported passively by convection currents Gräff (34), too, thought chemotaxis to be dependent on a hydrogen ion gradient, but other workers have found no effect of hydrogen and hydroxyl ions on chemotaxis (80) In this connection it is interesting to note that myxomycetes (slime molds) exhibit negative chemotaxis to adequate concentrations of both hydrogen and hydroxyl ions (17)

In the older literature, reviewed by Wells (85), are found reports on the chemotactic effects of a wide variety of substances These will not be reviewed in the present paper

*F Conditions interfering with or lessening chemotaxis* are important from the standpoint of the defense against infection, since, if cells cannot be attracted,

their arrival in an area of infection will be delayed, and thus bacteria have a better opportunity to become established As already stated, bacteria of high virulence may inhibit the motion of leukocytes. While the effect of poisoning of leukocytes is chiefly on their rate of locomotion, there is evidence from other types of experiment that such poisoned cells show also decreased chemotaxis. Thus in the case of individuals who were acutely ill from a variety of causes, their leukocytes in vitro showed both decreased rate of locomotion and less directional response to bacteria (56)

In acute experimental intoxication with alcohol (Klepser and Nungester, 44), the same functions of leukocytes were depressed the rate of locomotion was decreased and the cells, in the presence of pneumococci, showed no chemotactic response With the slide-coverslip method employing whole blood of rats, the chemotactic index in leukocytes from normal animals was +0.55, in cells from animals comatose from alcohol, the value was +0 06 Thus chemotaxis was abolished When alcohol was added directly to human blood *in vitro*, the chemotactic index fell from a control value of +0 46, to +0.24 when the concentration of alcohol was 0 1 per cent, and to +0 16 with an alcohol concentration of 0 4 per cent Such effects of alcohol may be responsible in part for the decreased resistance to infection occurring in alcoholic intoxication

G *Relation of chemotaxis to emigration of leukocytes* Emigration of leukocytes from blood vessels is widely regarded as the result of chemotaxis, in the sense that a concentration gradient of an attracting substance exists between inflamed tissue outside the vessels and the leukocyte inside the vessel, and as the result, the leukocyte moves through the vessel wall into the outside tissue Against this explanation is the fact that one of the best ways to cause leukocytes to emigrate is to inject a physiological salt solution into a serous cavity Although such a solution when tested *in vitro* has no chemotactic effect, it causes vast numbers of leukocytes to collect in serous cavities Thus emigration apparently may occur without chemotaxis But, it should be added, if the salt solution contains a chemotactic substance even greater numbers of leukocytes emigrate (16) As another example of emigration due to factors other than chemotaxis, lymphocytes have been observed to emigrate from vessels (12), although they are apparently incapable of displaying chemotaxis. A clearer understanding of the factors in emigration is gained from the experiments of Clark and Clark (13), who studied the reactions of leucocytes in the transparent tail and fin of the tadpole, and in the transparent chamber set in the rabbit ear If slight mechanical pressure was made on a blood vessel, leukocytes were seen to adhere to the endothelium for a short time, and then usually were carried away by the blood stream Sticking appeared to result from reversible changes in the vascular endothelium Greater pressure, or injury to the surrounding tissues, however produced, was followed by adhesion of many cells to the vessel wall, and by morphological changes in the lining cells that were interpreted as softening Some of the leukocytes adherent to the endothelium then passed through the vessel wall by amoeboid motion Thus emigration was always preceded by changes in the endothelium, interpreted as increased stickiness and

as softening, so that emigration appeared to result not from chemotaxis but from endothelial changes / According to Baron and Chambers (3) the leukocytes, too, become sticky

Various other mechanisms have been proposed to explain emigration of leukocytes (45), all depending on differences in physical or chemical factors between the blood inside vessels and the tissues outside vessels These factors have been thought to be the pressure exerted by the blood on the vessel wall, higher concentration of hydrogen ions (31) outside the vessels than inside, or greater viscosity of tissues as compared with blood (32) All these hypotheses lack confirmation, as does the more recently expressed view of Abramson (2) that emigration may be brought about by differences in electric potential between the injured area and the blood From evidence already presented it seems likely that emigration is conditioned first by changes in endothelium with consequent stimulation of leukocytes to display amoeboid movement and to pass through the vessel wall, second, emigration is probably increased by chemotaxis

*H The mechanism of chemotaxis* The generally accepted explanation of the phenomena of chemotaxis in leukocytes is the surface tension theory, as developed by Wells (85) He regarded the leukocyte as a drop of viscous liquid, which tends to take a spherical shape If one side of the cell should be exposed to some substance that lowers tension at the surface of the leukocyte, the cytoplasm would flow out in that direction and the cell move Thus the cell is regarded as a passive drop of liquid, under the control of surface forces, a conception that arose as the result of experiments with artificial cells (73) A number of cell models have been contrived which imitate certain aspects of amoeboid motion Thus if a drop of mercury is surrounded by a dilute solution of nitric acid, and a crystal of potassium dichromate is placed near the drop of mercury, chemical changes occur especially on that part of the surface of the mercury nearest the crystal, and the drop of mercury suddenly and rapidly moves to the crystal and flows around it Thus positive chemotaxis is simulated (4) If the mercury is placed in a solution of potassium dichromate, and nearby is placed a drop of concentrated nitric acid, the mercury is impelled to move in the opposite direction, that is, away from the nitric acid, and thus negative chemotaxis is simulated (19)

This conception of the leukocyte as a drop of liquid behaving passively is not in accord with present day conceptions of the structure and mode of motion of the cell, for amoeboid motion is now explained by most workers as the result of reversible sol-gel transformations of the cytoplasm The forward end of the cell is regarded as chiefly in the sol state, and this sol becomes transformed to gel at the surface as the cell moves The rear end of the cell is mostly in the gel state, the gel is transformed to sol, which is pushed forward by contracting jelled cytoplasm, either because the gel is under tension (59) or because it contracts much as does muscle (47) Thus the motion of the cell is not passive but active, involving transformation of chemical energy into kinetic

Chemotaxis is a directional response superimposed on amoeboid motion as just outlined In fact, amoeboid motion takes place just as well without chemotaxis

as with it, the rate of locomotion not being increased by the chemotactic response (25) What chemotaxis does is apparently to determine where in the cell shall occur solation and where gelation. How this is done is unknown Evidently the cell is able to detect differences in concentration of the exciting substance at the one end of the cell and at the other Polymorphonuclear leukocytes at least, are able to detect this difference when they lie in a concentration gradient of a chemotactic substance, whereas lymphocytes, at least according to present information, lack a mechanism for responding to difference in concentration of substances in their environment

If the simple surface tension theory of chemotaxis were correct, there should be closer correspondence between chemotaxis and phagocytosis than we find. Thus there is evidence that phagocytosis is promoted by, or, indeed, may be dependent on certain surface active substances, namely, antibodies (68) These are thought to coat bacteria and to lower the tension at the leukocyte-bacterial interface, so that the leukocyte is able to spread upon and to engulf the bacterium Here is an example of the effect of surface active substance upon phagocytosis. If chemotaxis also were under the influence of surface active substances, then antibodies should increase chemotaxis, but available evidence is opposed to this supposition When leukocytes were observed by the slide-coverslip technic, and their direction was followed in the presence of streptococci, specific antibodies were found not to increase chemotaxis to the bacteria, whether the leukocytes were attracted strongly or weakly, whether the antibody was adsorbed to the bacteria or was contained in the plasma medium (20)

It is concluded that the weight of evidence is against the surface tension explanation of chemotaxis, as it was elaborated by Wells On the other hand, no complete and satisfactory alternative theory has as yet been proposed.

*I The relation of chemotaxis to phagocytosis* The idea seems to be held by many pathologists that phagocytosis is always preceded by chemotaxis, that is, before a bacterium can be engulfed, it must attract the phagocyte from a distance. This is certainly not correct Many particles, especially of inorganic material such as carbon and silica do not attract leukocytes though they are frequently phagocytized by them as the result of chance encounters (30) This may readily be observed in slide-coverslip preparations, where polymorphonuclear leukocytes may accidentally make contact with such particles and may then ingest them Monocytes, the most voracious phagocytes, are, at least in mammals, not definitely known to be attracted to the bacteria and cells that they engulf (18) Thus it is clear that phagocytosis is not necessarily preceded by chemotaxis Conversely, chemotaxis is not always followed by phagocytosis, as was seen when polymorphonuclear leukocytes were attracted by dead cells, droplets of cream and of egg yolk, yet did not engulf these objects (14) A still more impressive example of chemotaxis not followed by phagocytosis is the negative, i.e., repelling, effect of silicates, described above (52)

The mechanisms of phagocytosis and of chemotaxis appear to be quite different. Phagocytosis depends on the ability of the leukocyte to spread on the object to be phagocytized, and spreading is greatly aided by specific antibodies known as

tropins (68) Tropins, as noted above, appear to play no part in chemotaxis which is apparently a non-specific reaction excited often by antigens, but not so far as is known, by antibodies (20) [1] Chemotaxis is not essentially a spreading reaction, like phagocytosis, but a reaction that depends on complicated sol-gel transformations in different parts of the cell body

*J Of what advantage is chemotaxis of leukocytes* to the organism and under what circumstances does this reaction occur? It has generally been accepted that leukocytosis, i e , increased numbers of leukocytes in the blood, depends on chemotactic attraction of leukocytes from the bone marrow into the blood stream For this supposition there seems to be no evidence It has likewise been held by most pathologists that emigration of leukocytes from blood vessels into tissues is the result of chemotaxis This has already been discussed, and the situation has been summarized by saying that both chemotaxis and changes in vascular endothelium probably play a part ✓Chemotaxis is chiefly important as the reaction by means of which leukocytes are attracted to infecting microorganisms *from a distance It is especially the polymorphonuclear leukocytes that are so* attracted These cells constitute the first line of cell defense They are mobilized quickly and in great numbers By chemotaxis they are directed in more or less straight lines toward bacteria, and thus much less time is required to bring these leukocytes into actual physical contact with the microorganisms This contact is followed, if the bacterial surface is suitable, by phagocytosis and intracellular digestion By means of these successive activities of the leukocyte, infection may be overcome Chemotaxis, phagocytosis and intracellular digestion are thus consecutive activities of the leukocyte depending on different mechanisms

## REFERENCES

(1) ABELL, R G Personal communication to the author
(2) ABRAMSON, H A J Exper Med 46 987, 1927, J Gen Physiol 11 743, 1923
(3) BARON, H AND R CHAMBERS Am J Physiol 114 700, 1936
(4) BERNSTEIN, J Pflüger's Arch 80 628, 1900
(5) BLOCH, G Centralbl f allg Path 7 785, 1896
(6) BLOOM, W Bull Johns Hopkins Hosp 33 185, 1922
(7) BUCHNER, H Berl klin Wchnschr 27 673, 1890
(8) CHAMBERS, R AND C G GRAND J Cell and Comp Physiol 8 1, 1936
(9) CHAMBERS, R AND C G GRAND Proc Soc Exper Biol and Med 36 673, 1937
(10) CLARK, E R AND E L CLARK Am J Anat 27 221, 1920
(11) CLARK, E R AND E L CLARK Anat Rec 24 137, 1922
(12) CLARK, E R AND E L CLARK Am J Anat 46 149, 1930
(13) CLARK, E R AND E L CLARK Am J Anat 57 385, 1935
(14) CLARK, E R , E L CLARK AND R O REX Am J Anat 59 123, 1933
(15) COMAN, D R Am J Med Sci 196 273, 1938
(16) COMAN, D R Am J Path 15 597, 1939
(17) COMAN, D R Arch Path 29 220, 1940
(18) COMAN, D R Arch Path 30 896, 1940
(19) COMAN, D R Personal communication to the author
(20) COMAN, D R , M McCUTCHEON AND P T DECAMP Proc Soc Exper Biol and Med 41 119, 1939

[1] Opie (69) found that the precipitate formed by interaction of horse serum and anti horse rabbit serum is chemotactic for leukocytes when injected into the skin of the rabbit

1) Comandon J Compt rend Soc de Biol 80: 314, 1917
2) Comandon J Compt rend Soc de Biol 82: 1171 1919
3) Dixon H M and M McCutcheon Arch Path 19 679 1935
4) Dixon, H M and M McCutcheon Am J Path 11: 872 1935
5) Dixon, H M and M McCutcheon Proc Soc Exper Biol and Med 34: 173, 1936
6) Dixon, H M and M McCutcheon Proc Soc Exper Biol and Med 38: 378, 1938
7) Dixon H M, M McCutcheon and E J Czarnetsky Am J Pathol 13: 645 1937
8) Duthie E S and E Chain Brit. J Exper Path 20: 417 1939
9) Emys Roberts, E and E M Cowell. J Path and Bact 21: 473 1917
0) Fenn W O J Gen Physiol 5 311 1923
1) Feringa K J Pflüger s Arch 203 663 1924
2) Friedemann, U and A Schönfeld Biochem Ztschr 80: 312 1917
3) Gabritchevsky G Ann Inst Pasteur 4: 346 1890
4) Graff S Münch med Wchnschr 69: 1721 1922
5) Grand C G and R. Chambers J Cell and Comp Physiol 9 165, 1936
6) Grant R T and J E Wood J Path and Bact 31: 1 1928
7) Haan, J de Arch néer Physiol 2: 674, 1918
8) Häbler C and C Weber Klin Wchnschr 1: 760, 1930
9) Ingraham, E S and W B Wartman Arch Path 28: 318, 1939
0) Jacoby F J Physiol 91 22P 1937–8
1) Jennings H S Behavior of the lower organisms New York Columbia University Press 1931 See also Hyman L H The invertebrates Protozoa through Ctenophora New York, McGraw Hill Book Company, 1940 p 67
2) Jochims, J Pflüger s Arch 216 611 1927
3) King J T B S Green and A F Henschel. Proc Soc Exper Biol and Med 38 812, 1938
4) Klepser R. G and W J Nungester. J Infect Dis 65: 196 1939
(45) Krehl, L and F Marchand Handbuch der Allgemeine Pathologie Leipzig, 1924 S Hirzel vol 4 part 1 p 329
(46) Leber, T Fortschr d Med 6 460 1888 cited by H G Wells (85)
(47) Lewis, W H Arch f exper Zellforsch 23 1 1939
(48) Lidforss B Ztschr f Botanik 1: 443 1909
(49) Lurie M B J Exper Med 60 163 1934
(50) McCutcheon, M Am J Physiol 66 180 1923
(51) McCutcheon M Am J Physiol 66: 185 1923
(52) McCutcheon M D R. Coman and H M Dixon Arch Path 27: 61 1939
(53) McCutcheon M and H M Dixon Arch Path 21 749 1936
(54) McCutcheon, M and H M Dixon Unpublished experiments
(55) McCutcheon M W B Wartman and H M Dixon Arch Path 17 607 1934
(56) Mallery, O T., Jr and M McCutcheon Am J Med Sci 200 394 1940
(57) Massart J Ann Inst Pasteur 6 321 1892.
(58) Massart, J and C Bordet J de Méd 90 169, 1890
(59) Mast S O Physiol Zool 5 1, 1932
(60) Meier, R. Ztsch Exper Med 87: 283 1933
(61) Meier, R Helvetica Chim Acta 24 (Suppl ) 134E, 1942
(62) Menkin V J Exper Med 67: 129 145 1938
(63) Menkin V J Exper Med 67: 153 1938
(64) Menkin V Dynamics of inflammation New York The MacMillan Company, 1940 p 59
(65) Mitoshi M Flora 78: 76 1894
(66) Moon, V H Arch Path 20 561 1935
(67) Morgan H R and H C Upham Proc Soc Exper Biol and Med 43: 114 1941
(68) Mudd S M McCutcheon and B Lucké Physiol Rev 14: 210 1934
(69) Opie E L J Immunol 9: 259 1924
(70) Pettersson A Centralbl f Bakt (Abt 1) 103 294 1926

(71) PFEFFER, W Untersuch a d bot Inst zu Tübingen 1 363, 1884
(72) PFOEHL, J Centralbl f Bakt 24 343, 1898
(73) RHUMBLER, L In E ABDERHALDEN Handbuch der biologischen Arbeitsmethoden, Berlin, Urban & Schwarzenberg, 1923, vol 5, sect 3, pt 2, p 219
(74) ROBERTSON, O H Physiol Rev 21 112, 1941
(75) ROSEGGER, H Ztschr exper Med 85. 712, 1932
(76) RUCHLÄDEW, N Ztschr f Biol 54 533, 1910
(77) SCHADE, H AND K MAYR Krankheitsforschung 8 354, 1930
(78) SHELDON, W F Personal communication to the author
(79) SICHERER, O v Centralbl f Bakt 26 360, 1899
(80) SILVERMAN, D Arch Path 25 40, 1938
(81) STEVENSON, J W AND G B REED J Bact 40 239, 1940
(82) Tabulae biol 4 356, 437, 1927
(83) WARTMAN, W B Arch Path 26 694, 1938
(84) WARTMAN, W B AND E S INGRAHAM Arch Path 29 773, 1940
(85) WELLS, H G Chemical pathology Ed 5, Philadelphia, W B Saunders Company, 1925
(86) WOLF, E P J Exper Med 34· 375, 1921
(87) WOODRUFF, C E Am J Path 10· 739, 1934

# THE PHYSIOLOGY OF NERVOUS SYSTEMS OF INVERTEBRATE ANIMALS

C LADD PROSSER

*Department of Zoology and Physiology University of Illinois Urbana*

The basic metabolic functions of an animal can proceed without any highly specialized conducting tissue The specialization of conducting tissue, particu larly of nervous tissue, gives an animal a certain freedom from the environment which has survival value, this freedom permits a combination of large size and high motility Many Protozoa and larvae are small and have little rapid conduction from one part to another, yet are motile Many sessile animals, on the other hand, have slightly developed conducting mechanisms, they may be large but they are not motile All of those animals which are both large and motile have rapidly conducting nervous tissue

In the evolution of nervous tissues four trends are apparent (1) increasing speed of conduction, (2) integration, or making one impulse count for more or less than one, (3) cephalic dominance, and (4) chemical mediation of nerve impulses The present review aims to discuss the attainments of invertebrate animals in each of these directions of nervous evolution and to point out useful preparations for physiology rather than to chronicle the literature on invertebrate nervous systems The most recent review of this subject was by Ten Cate in 1931 (301)

PRE-NERVOUS CONDUCTION Among the animals which do not have nervous systems some co-ordinating mechanisms have been described Probably all living cells have the ability to conduct waves of excitation, and conduction in many plant cells, eggs and body cells of higher animals has been described as a wave of depolarization passing along the plasma membrane. Conduction from cell to cell, as in ciliated epithelium, is well known Conduction by way of many intracellular fibrils has been claimed for a few Protozoa

Taylor recently reviewed the protozoan fibrillar systems (292) Many ciliates have a regular network of fibrils connecting the basal granules of the cilia The form of this network varies, but in general the basal granules are connected in longitudinal rows with some cross-connections (115 and others) This peripheral network is connected to the central neuromotor system which may have a center or motorium and has internal fibrils which extend to mem branelles, cytopharynx, and cirri where these are present The entire fibrillar system has been variously claimed to be supporting, contractile and conducting Experimental evidence of a conducting function came from Taylor (291), who with microneedles cut fibrils between the motorium and the cirri in Euplotes (and cut the peripheral fibrils as well), co-ordination between the cirri and membranelles in the oral region disappeared. Disruption of co-ordination of membranelles occurred after destruction of the motorium in Chlamydodon (188), and disorganization of ciliary movement after fibrillar injury in Ichthyo phthirus (190) However, in Paramecium lesions near the peristome did not

destroy ciliary co-ordination (204), even though a motorium is said to be located there (187)

Conduction in higher animals is a function of cell membrane and even in neurones the fibrils per se probably have nothing to do with conduction Taylor (292) suggests that the fibrillar system in ciliates actually may have several different functions Ciliates are highly specialized acellular organisms Hence, the ciliate fibrillar system is not a precursor of nervous structures

In sponges there are no nerve cells, but spindle-shaped muscle cells bring about closure of oscula These muscle cells Parker (229) has called independent effectors because they combine sensory and motor functions There is a slow conduction (about 1 cm /min ) from cell to cell in a sponge as shown by the response of the osculum to a prick a short distance away (223) In some coelenterate tentacles there may be a few two-celled arcs (sense cell—muscle cell) (229), but beginning with the coelenterates co-ordination between sense organs and effectors is essentially nervous

NERVOUS CONDUCTION *Action Potential Wave* Impulses in nerve fibers of invertebrate animals consist, as in vertebrates, of several electrical components The non-medullated fibers of the squid (6) and crab (136) have been used for the demonstration of non-propagated, graded, local potentials From the local potential emerges the propagated spike, which is followed by after-potentials In crab nerve a negative "retention" may continue for fifteen minutes after a stimulus (182, 110) Zhukow (337) described an after-positivity which might persist for a similar time Veratrine caused the after-negativity in crab fibers to continue for a half-hour (79, 27), and the after-negativity was lengthened by repetitive stimulation (46) These invertebrate nerves provide excellent preparations for analysis of the cellular basis for after-potentials

*Speed of Conduction* Five different adaptations of nerves have provided increased speed of reaction among animals length of nerve processes, fiber diameter, myelin sheath, nodes, and giant fibers

(*1*) *Length of nerve-processes* The first nervous adaptation by which animals increase their speed of reaction is the lengthening of conducting processes If a message has to pass through numerous synapses, with a delay at each, it travels more slowly than if it can go by way of a single fiber In many coelenterates, for example, the nervous system consists of a network of multipolar and bipolar neurones whose processes have been traced for several millimeters (50) Conduction in this network is slow, less than 0 5 m /sec (table 1) In some coelenterates through tracts of continuous fibers exist for rapid conduction (221) In general, fast-moving animals have some nerve tracts consisting of long single fibers Giant fiber conduction is more rapid than conduction through the neuropile in ganglion chains of annelids and arthropods (table 1)

(*2*) *Fiber diameter* In mixed nerves of frogs and mammals speed of conduction is proportional to a function of the fiber diameter The fiber-diameter/velocity correspondence has not been investigated for the entire spectrum of any invertebrate nerves However there is no doubt that faster animals have some large motor nerve fibers, and in slower animals most nerve fibers are small

In some mammalian fibers the velocity in meters per second numerically equals the diameter times a constant (6 or 7) (113, 149), in frog fibers the velocity varies as the square of the diameter (94), and in giant fibers of the squid velocity increases as the diameter raised to the 0 61 power (251)

TABLE 1

*Velocity of conduction in ganglionic cords and nerve nets*

| ANIMAL | REFERENCE | TEMPERATURE | VELOCITY |
|---|---|---|---|
| **Ganglionic cords** | | | |
| | | °C | *m p.s* |
| Annelids | | | |
| Earthworm | (181) | | 0 6 |
| Leech | (181) | | 0 4 |
| Earthworm (Helodrilus) | (48) | | 0 02 –0 03 |
| *Cerebratulus | (155) | | 0 059–0 09 |
| *Arenicola | (155) | | 1 2 |
| *Aphrodite | (155) | | 0 545 |
| Myriapods | | | |
| Scolopendra ant → post | (62) | | 2 5 |
| Scolopendra post → ant | (62) | | 2 5 |
| Julus ant → post | (62) | | 0 2 |
| H mantarium ant → post | (62) | | 0 28 |
| Crustacea | | | |
| Cambarus | (243) | 20–24 | 1 2 |
| **Nerve nets of coelenterates**** | | | |
| Metridium | (228) | 21 | 0 121–0 146 |
| Calliactis (net) | (221) | | 0 04 –0 15 |
| Calliactis (through tracts) | (221) | | 1 2 |
| Physalia filaments | (231) | 20 | 0 121 |
| Cassiopea | (120) | 18 | 0 136–0 234 |
| Cassiopea | (190) | | 0 15 –1 2 |
| Mastigias | (170) | 25 5 | 0 57 |
| Renilla (muscular wave) | (230) | 21 | 0 078 |
| Renilla (luminescent wave) | (230) | 21 | 0 074 |

* These are a few of the numerous values given by Jenkins and Carlson (155) Some of the species studied are now known to have giant fibers and some of the higher velocities they recorded may have been in giant fibers

** Most conduction velocities in coelentorates are measured as speeds of contractile waves rather than as conduction in nerve net only

Table 2 gives the velocities in moist air and where possible the total fiber diameters in a variety of nerve fibers The general relationship between diameter and velocity holds The motor fibers of the legs of crustacea for example are 20–30 $\mu$ in diameter and conduct at 1.5–12 m /sec while molluscan fibers about 1 $\mu$ in diameter conduct at less than 1 m /sec. The most striking adaptation of

TABLE 2

*Velocity of conduction in representative nerve fibers as related to fiber diameter and sheath thickness*

| ANIMAL | NERVE | REFERENCE | FIBER DIAMETER (MICRA) | AXIS CYLINDER DIAMETER/ FIBER DIAMETER | TEMPERATURE | VELOCITY |
|---|---|---|---|---|---|---|
| **Warm blooded vertebrates** | | | | | | |
| Dog | saphenous | (113) | 17 | | 38 | 83 3 |
| Dog | saphenous | (295) | 16 | 0 82 | | |
| Dog | saphenous | (113) | 14 4 | | 38 | 71 5 |
| Dog | n lingualis | (113) | 13 5 | | 38 | 69 1 |
| Cat | saphenous | (114) | 13 | 0 71 | 38 | 80 |
| Cat | saphenous | (149) | 12–15 | | 37 5 | 65–82 |
| Cat | saphenous | (114) | 9 | 0 69 | 38 | 60 |
| Dog | chorda tympani | (113) | 8 2 | | 38 | 36 4 |
| Dog | saphenous | (114) | 5 | 0 6 | 38 | 20 |
| Cat | saphenous | (295) | 2 | 0 3 | | |
| Rabbit | depressor | (217) | 4–2 | | 38 | 5 |
| Cat | sympathetic | (42) | thin | non myelinated | 38 | 2–1 |
| **Cold blooded vertebrates** | | | | | | |
| Frog | dorsal root | (113) | 18 5 | | 21 5 | 42 |
| Frog | dorsal root | (113) | 14 | | | 25 |
| Frog | dorsal root | (113) | 11 | | | 17 |
| Frog | dorsal root | (113) | 5 8 | | | 4 2 |
| Frog | sciatic | (274) | 6–16 | 0 7 | | |
| Frog | sciatic | (274) | 2–3 | 0 5 | | |
| Frog | B fibers | (42) | small | thinly myelinated | | 4 5–3 |
| Frog | dorsal root (C fibers) | (113) | 2 5 | non myelinated | | 0 5–0 4 |
| Turtle | sympathetic & vagus | (42) | | non myelinated | | 0 8–0 3 |
| **Invertebrates** | | | | | | |
| Arthropods | | | | | | |
| Maia | leg nerve | (186) | 10–20, 4–8, 1–3 | | 23–25 | 2 5–3 7<br>1 1–1 75<br>0 1–0 5 |
| Carcinus | leg nerve | (137) | 30 | very thin sheath | 21 | 3.5 in oil |
| Giant crab | leg nerve | (165) | | | | 2 7, 3 9 |
| Callinectes | leg nerve | (205) | | | | 4.8, 1.5 |
| Munida | leg nerve | (142) | 50 | very thin sheath | 17 | 6 4 |
| Homarus | leg nerve | (205) | | | | 9.2, 1.8 |
| Homarus | leg nerve | (63) | | | | 12 |
| Cambarus | leg nerve | (88) | | | | 4.2, 3 |
| Leander | | (142) | <10 | 0 53 | | |
| Leander | | (142) | 10–20 | 0 69 | | |
| Limulus | leg nerve | (205) | | | | 4 6, 1.3 |
| Cockroach | cercal nerve | (250) | | | | 5–6, 1.5 |
| Cockroach | central fibers | (256) | 10–20 | 0 9–0.95 | | |
| Molluscs | | | | | | |
| Helix | n. intestinalis | (178) | (<1 ?) | | | 0 4 0.05 |
| Arolimax | pedal | (154) | | | 15–18 | 0 41 |
| Limax | pedal | (154) | | | | 1 4 |
| Pleurobranchiae | pedal | (154) | | | 12–15 | 0 78 |
| Mytilus | pedal | (258) | | | | 0 64 |
| Sepia | mantle | (46) | <50 | | | 3.5, 2.26 |
| Sepia | fin | (46) | | | | 5 1 |
| Sepia | stellar | (7) | | | | 3, 1.5, 0.32, 0.22 |
| Loligo | | (251) | 42 6 avg | | 21-22 | 4.6 avg |
| Loligo | fin | (154) | | | | 4 3 |

TABLE 2—*Concluded*

| ANIMAL | NERVE | REFERENCE | FIBER DIAMETER (MICRA) | AXIS CYLINDER DIAMETER/FIBER DIAMETER | TEMPERATURE | VELOCITY |
|---|---|---|---|---|---|---|
| | | | Giant nerve fibers | | | |
| Vertebrate | | | | | | |
| Amphioxus | Mauthner fibers | (117) | 22–43 | 0.59–0.86 | 10–15 | 50–60 |
| Arthropod | | | | | | |
| Cambarus | | (319) | 200–220 (median) 60–80 (lateral) | | | |
| Lumbricus | | (142) | 35 | 0.77 | 17 | 15–23 in oil |
| Maia | | (142) | 80 | thin sheath | 17 | 6.4 |
| Shrimp | | (294) | >35 <8 | 0.87 metatropic | | |
| Annelid | | | | | | |
| Lumbricus | | (293) | >40 <11 | 0.90 metatropic | | |
| Lumbricus | | (59) | median lateral | | 10–12 10–12 | 17–25 7–12 |
| Lumbricus | | (285) | median 64 lateral 37 | | | |
| Lumbricus | | (85) | median lateral | | | 15–45 8–18 |
| Lumbricus | | (43) | | | | 1.8 |
| Lumbricus | | (259) | | | | 30–10 |
| Mollusc | | | | | | |
| Sepia | | (281) | 16" | | | 7–7 |
| Loligo | | (281) | 718 559 avg. 335 avg. | | | 22.3 20.6 13.7 |
| Loligo | | (276) | 250–400 | | 22 | 20 |
| " | | (29) | 400–500 | >0.99 metatropic | | |

fiber diameter to velocity of conduction is in the giant fibers of crustaceans, cephalopods and annelids. These may have diameters of tens of microns or several hundred microns and conduct many times faster than the smaller fibers of these animals. Insects in general have very small nerve fibers but conduction distances are short, hence fast conduction is less important than a large number of fibers in a small volume. Richards, for example, states (256) that mosquito larvae fibers are less than 1–2 $\mu$ although cockroaches have some 10 $\mu$ fibers.

Excitation times also vary inversely with fiber diameters, i.e., fast fibers have short chronaxies.

(5) *Nerve fiber sheath.* In addition to fiber diameter the existence of a lipoid sheath around the axon is directly correlated with velocity of conduction. Among vertebrates the larger fast motor and sensory fibers have myelin sheaths which blacken with osmic acid and can be readily measured, while the slow small sympathetic fibers are said to be non myelinated. When examined in polarized light the larger fibers are seen to be negatively birefringent with respect to their long axis, this is due to the lipid sheath and the fibers are called myelotropic. The birefringence in frog fibers is nearly constant among fibers 9 $\mu$ or more in diameter but diminishes in smaller fibers until it is zero at 2 $\mu$ and below that it reverses sign (274). The birefringence of the "non medullated" fibers on the contrary is normally positive with respect to length, is due to proteins, and can

TABLE 2

*Velocity of conduction in representative nerve fibers as related to fiber diameter and sheath thickness*

| ANIMAL | NERVE | REFERENCE | FIBER DIAMETER (MICRA) | AXIS CYLINDER DIAMETER/ FIBER DIAMETER | TEMPERATURE | VELOCITY |
|---|---|---|---|---|---|---|
| **Warm blooded vertebrates** | | | | | | |
| Dog | saphenous | (113) | 17 | | 38 | 83 3 |
| Dog | saphenous | (295) | 16 | 0 82 | | |
| Dog | saphenous | (113) | 14 4 | | 38 | 71 5 |
| Dog | n lingualis | (113) | 13 5 | | 38 | 69 1 |
| Cat | saphenous | (114) | 13 | 0 71 | 38 | 80 |
| Cat | saphenous | (149) | 12–15 | | 37 5 | 65–82 |
| Cat | saphenous | (114) | 9 | 0 69 | 38 | 60 |
| Dog | chorda tympani | (113) | 8 2 | | 38 | 36 4 |
| Dog | saphenous | (114) | 5 | 0 6 | 38 | 20 |
| Cat | saphenous | (295) | 2 | 0 3 | | |
| Rabbit | depressor | (217) | 4–2 | | 38 | 5 |
| Cat | sympathetic | (42) | thin | non myelinated | 38 | 2–1 |
| **Cold blooded vertebrates** | | | | | | |
| Frog | dorsal root | (118) | 18 5 | | 21 5 | 42 |
| Frog | dorsal root | (113) | 14 | | | 25 |
| Frog | dorsal root | (113) | 11 | | | 17 |
| Frog | dorsal root | (113) | 5 8 | | | 4 2 |
| Frog | sciatic | (274) | 6–16 | 0 7 | | |
| Frog | sciatic | (274) | 2–3 | 0 5 | | |
| Frog | B fibers | (42) | small | thinly myelinated | | 4 5–3 |
| Frog | dorsal root (C fibers) | (113) | 2 5 | non myelinated | | 0 5–0 4 |
| Turtle | sympathetic & vagus | (42) | | non myelinated | | *0 8–0 3* |
| **Invertebrates** | | | | | | |
| Arthropods | | | | | | |
| Maia | leg nerve | (186) | 10–20, 4–8, 1–3 | | 23–25 | 2 5–3 7<br>*1 1–1 75*<br>0 1–0 5 |
| Carcinus | leg nerve | (137) | 30 | very thin sheath | 21 | *5.5 in oil* |
| Giant crab | leg nerve | (165) | | | | 2 7, 3 9 |
| Callinectes | leg nerve | (205) | | | | 4.8, 1.5 |
| Munida | leg nerve | (142) | 50 | very thin sheath | 17 | 6 4 |
| Homarus | leg nerve | (205) | | | | *9.2, 1.8* |
| Homarus | leg nerve | (63) | | | | 12 |
| Cambarus | leg nerve | (88) | | | | 4.2, 3 |
| Leander | | (142) | <10 | 0 53 | | |
| Leander | | (142) | 10–20 | 0 69 | | |
| Limulus | leg nerve | (205) | | | | 4 6, 1.3 |
| Cockroach | cercal nerve | (250) | | | | 5–6 1.5 |
| Cockroach | central fibers | (256) | 10–20 | 0 9–0.95 | | |
| Molluscs | | | | | | |
| Helix | n intestinalis | (178) | (<1 ?) | | | 0 4 0.05 |
| Ariolimax | pedal | (154) | | | 15–18 | 0 41 |
| Limax | pedal | (154) | | | | 1 4 |
| Pleurobranchiae | pedal | (154) | | | 12–15 | 0 78 |
| Mytilus | pedal | (258) | | | | 0 64 |
| Sepia | mantle | (46) | <50 | | | 3.5, 2.25 |
| Sepia | fin | (46) | | | | 5 1 |
| Sepia | stellar | (7) | | | | 3, 1.5, 0.32, 0.22 |
| Loligo | | (251) | 42 6 avg | | 21-22 | 4.6 avg |
| Loligo | fin | (154) | | | | 4 3 |

type consists of a syncytium in which processes from a number of nerve cells fuse to form a single giant axon Unicellular giant fibers are found in nemerteans and cestode worms, in numerous polychaetes, in balanoglossids and in lower vertebrates In the polychaete Halla for example (8) are groups of unipolar giant cells 30–150 $\mu$ in diameter in each of the first few segments, these give rise to fibers up to 40 $\mu$ thick which may run the length of the worm Similar unicellular giant fibers have been described in other worms Bullock (57) and Hess (135) have described the giant fiber system in a number of balanoglossids Typically there are about a dozen to 150 giant cells, usually in the collar nerve cord, these give rise to large (3–6 $\mu$) fibers which run back in the nerve cord and eventually dwindle in size to merge with the ordinary fibers ($< 1 \mu$) The only functional studies on the unicellular type of giant fiber are on the Mauthner and Müller cells which occur in the tegmental nuclei (part of the vestibular reflex system) of fishes and amphibians These fibers, 22–43 $\mu$ in diameter, conduct at 50–60 m /sec. at 10–15°C (table 2) (117)

The multicellular or syncytial type of giant fiber system has been described in annelids, crustacea and cephalopod molluscs

In the ventral nerve cord of the earthworm there are in each segment several cells which send axones to the three dorsal giant fibers, one large median and two smaller lateral ones These giant fibers run the full length of the ventral nerve cord and give off segmental branches to motor neurones in the cord The median giant fiber is connected to two pairs of giant cells and each lateral giant to one pair of giant cells per segment (289) The lateral giant fibers are also connected with each other In addition to the three dorsal giant fibers there are two smaller ventral ones (288, 280) At each segment there is in the giant fibers an oblique partition the two sides of which stain differently (289, 58) This was thought by Stough to be a macrosynapse

Evidence from cutting the giant fibers, from regeneration, from blocking by drugs, and from embryonic development indicates that in the earthworm the giant fibers are responsible for quick end to-end "startle" contractions (48, 239, 303, 289) Stough (289) cut single giant fibers and concluded that the median one conducts postero-anteriorly while the two lateral ones conduct antero-posteriorly Action potentials (89), however, showed that conduction is equally good in either direction in each fiber Bullock (58) settled the disagreement by showing that the median fiber is normally excited by sensory stimulation in the anterior forty segments while the lateral fibers are excited by sense organs behind that level The two lateral fibers and the median fiber form two separate conduction and excitability systems, cross connections between the lateral fibers were shown by conduction which continued when right and left fibers were alternately cut (209) The usefulness of the giant fiber system particularly in protective defense reactions is indicated by the fact that conduction in the smaller fibers of the cord (including synapses) is about 0 025 m /sec. (48) while in the lateral giant fibers it is 7–12 m /sec. and in the median one it is 17–25 m /sec. depending on fiber size (89) Multicellular giant fibers occur also in Nereis and in many other polychaete annelids

Among Crustacea giant fiber systems have been described in the crayfish, the lobster, and in the prawns Palaemonetes and Leander In the crayfish (157) there are three kinds of giant fiber median, lateral and motor The paired median giant fibers start in the brain (at least in the crayfish) decussate there and pass to the ventral nerve cord The lateral fibers occur in thoracic and abdominal ganglia and consist of segmental units which appear to overlap in long side-by-side contacts, they presumably are made up of processes from ganglion cells in each segment Branches are given off from the laterals and medians to motor giant fibers in each segment The motor fibers have their cell bodies in one segment, decussate, and pass out in the posterior nerve of the segment (157) Stimulation of the giant fiber system elicits quick flipping of the abdomen (158) and causes extension and inward movement of antennae (319) Johnson (157) thinks that the median ones conduct backward, the lateral ones forward We have found (Prosser and Altschul, unpublished) that the median and lateral giant fibers of the crayfish conduct equally well in both directions Wiersma (319) found summation between the two median giants to be maximum at an interval between stimuli of 1 7 msec and between the lateral giants at less than 4 5 msec , he noted some summation of the response of the median by stimulating the lateral giants and concluded that the four fibers govern the same nerve-muscle system

In the prawn Leander, Holmes (141) described two types of synapse, oblique regions of apposition between the lateral fibers in each segment and typical boutons at the points of contact of processes from both median and lateral with the motor fibers The median giant fibers showed incomplete septa Transection of the cord at various points failed to produce degeneration of the median or lateral giant fibers In Leander the motor giant fibers are non-myelinated from the cell body to their synapse and are myelinated from that point out to the muscles. The lipid sheath of the larger giants is interrupted occasionally (142) and is separated from the axon by a thin nucleated layer and does not have such a layer outside it as do the fibers in vertebrates The ratio of axis cylinder to total fiber diameter is 0 53 for fibers smaller than 10 $\mu$, 0 69 for 10–20 $\mu$ fibers and 0 77 for 20–50 $\mu$ fibers (142)

The cephalopod molluscs have a giant fiber system which is intermediate between the unicellular type and the segmentally fused type of giant fiber system The cephalopod system has been studied by Young (335, 336) In the decapods (squids) a pair of giant cells lie at the posterior end of the pedal ganglion portion of the brain, their processes fuse and cross, then synapse in the visceral part of the brain mass with giant cells whose processes run out in mantle nerves These synapse with a third set of giant fibers in the stellate or mantle ganglion From the stellate ganglion some dozen nerves pass out to the muscles of the mantle, each nerve contains one giant fiber which may be as much as 800 $\mu$ in diameter plus many one-micron fibers Each giant axon arises by the fusion of processes from 300–1500 cells in the stellar ganglia and the preganglionic axon ends by synaptic boutons near the region of fusion Each giant axon serves the circular muscles of a large area of the mantle and the largest fibers pass to the posterior

part of the mantle so that impulses arrive nearly simultaneously in the entire mantle (336) Velocities of conduction in fibers 30–718 $\mu$ in diameter range from 2.7 to 22.8 m /sec at 20° As shown in table 2 there is a very thin sheath Stimulation of a giant fiber elicits a maximal contraction at threshold (336), and there is no increase in tension with increasing frequency of stimulation (248), hence the one syncytial fiber with a large area of circular muscle is a motor unit The contractile response forces water out of the mantle cavity thus sending the animal backward, this escape reaction is, according to Young, twice as fast by virtue of the giant fiber system as it would be without it

Young (335) has described an interesting series among cephalopods In the decapod Sepia the cells contributing to the large giant fibers are scattered through the stellate ganglion In Loligo these cells are confined to one posterior lobe, while in the octopods Eledone and Octopus the lobe is present and contains cells which may resemble neurones but which lack true processes This lobe, the epistellar body, receives the secondary giant fibers from the visceral ganglion, that it may be a neurosecretory organ was indicated by an atonia of the mantle after removal of the epistellar body

Giant fibers have been very useful preparations for studying the membrane mechanisms of nerve conduction, and Curtis and Cole (80) have successfully recorded impulses with a fine electrode inserted down the center of a squid giant axon This method gives more nearly the total membrane potential than does the usual surface recording

Giant fibers, then, are usually adaptations of large fiber diameter for rapid escape reactions. Some are connected to one cell, some are syncytial. The syncytial annelid and crustacean fibers show either segmental septa or long areas of apposition which appear to conduct equally well in either direction Such junctions certainly do not behave physiologically in the same way as most synapses in higher animals The marked differences in structure and function indicate that giant fibers have been evolved independently in several groups

Co-ordination Among the co-ordinative functions which are associated with the vertebrate central nervous system are polarity of transmission, spatial and temporal summation, after-discharge and inhibition These, except for polarity, permit a single impulse to count for more or less than one. Among invertebrates much co-ordination occurs at neuromuscular junctions as well as in central nervous systems, and it is useful to consider both sites as parts of a common system.

*Nerve nets* Co-ordinative functions evolved very early, they are found among nerve nets where there is no condensed central nervous system Nerve nets or diffuse nervous systems have been studied functionally in coelenterates, both polyps and medusae and in ctenophores The elements of the nervous system of medusae were identified by Haeckel and the Hertwigs, and were described by a series of early histologists as a network of multipolar and bipolar neurones lying beneath the epithelium and connected to a condensed marginal ring Bethe (37) and Hadzi (121) contended that the nerve fibers anastomose and that the network is a syncytium Parker (229) also accepted this view Recent

evidence (50, 330) with vital stains shows that processes of the neurones often intertwine or run along parallel with one another and may have a beaded appearance while in contact but that the fibers do not fuse These "synapses" are not structurally polarized like vertebrate boutons but the nerve net is probably not a syncytium

Coelenterate nerve net systems show some co-ordinative characteristics

1 *Polarity* The first indications in medusae were of diffuse, non-polarized conduction (91, 266, 267) The marginal "sense organs" are the normal pacemakers for rhythmic contractions Conduction is diffuse and essentially equal in all directions If overlapping lateral and central incisions are made, or if the jellyfish is cut into a long zig-zag strip, or into a central disc connected by a narrow neck to an outer ring, impulses pass around corners and in both directions so long as nervous tissue is present That this diffuse conduction is nervous is shown by conduction through areas where muscles are absent, but nerve fibers present (200), in regenerating areas where conducting but not contracting tissue is present and in preparations treated with curare (266) or magnesium (267, 200, 56) A doughnut-shaped ring can be cut out of a jellyfish and a wave started in both directions from a stimulus at a point (199, 126), if one wave is blocked in some way, e g , by pressure on one side, a circusing self-perpetuating wave can be set up which keeps going around for many hours (up to several days), the same units are being re-excited repeatedly

Parker (227) made similar observations of diffuse conduction on sea anemones He made cuts of various shapes in the body wall and found that conduction went through various types of connection from the body wall to the muscles Similar conduction occurred from zooid to zooid in the colonial Renilla (230)

It is concluded from the effects of cuts in the nerve net and from the histological picture that there is no polarity in conduction from neurone to neurone Final evidence can come only when conduction across a single junction can be examined

The picture of a non-polarized diffuse conducting system is, however, too simple for all the facts Parker (227) showed that the oral disc of Metridium responds differently according to whether the tentacles are stimulated by acid or by mussel juice, indicating either specific functional tracts or differences in sensory response Pantin (222) showed that when a tentacle is removed from an anemone (Calliactis) or pieces cut from a tentacle the end of the central stump contracts while the end of the free piece remains open Also it is easier to get impulses out to the tentacles than in from them, i e , less facilitation is needed Pantin (221) also found some through conduction, especially in the mesenteries where velocities of 1 2 m /sec existed in contrast to local conduction around or up the anemone at 0 04–0 15 m /sec Through tracts were more important in Calliactis than in Anemonia Also the velocity of local conduction varied greatly in different regions This evidence from anemones indicates definite conduction pathways for different purposes and a sort of functional polarity Pantin indicated that this polarity may arise by the development of differential rates of facilitation

2. *Facilitation* It was formerly believed (162) that nerve net conduction is decremental because a contraction wave dies out with increasing distance from the stimulus Pantin has accounted for the decremental like conduction in sea anemones in terms of facilitation (220) He found that an anemone rarely showed any muscular response to a single shock but gave an increasing contraction as more stimuli were delivered. At low frequency (intervals greater than 2 sec ) a response might be seen only after 10–15 stimuli at 20° Increasing the intensity of successive shocks had little or no effect upon the contraction but increasing the frequency greatly increased the response to a maximum at 100–200 msec separation between stimuli. The second of two stimuli was effective to 5 per cent of the maximal response at 5° when separated from the first by 8 seconds, at 15° at 4 6 sec and 24° at 0 9 sec intervals (122) Each impulse excited some units subliminally, distant ones less than near ones, and new units were brought in as excitation built up The excitatory process persisted longer at low than at high temperatures Thus the response spread further and became stronger as more distant units were brought up to threshold

Bethe described facilitation in a tissue bridge in a medusa (39) and Bullock has studied facilitation in medusae in detail (56) Jellyfish differ from anemones in that single stimuli elicit a small contraction Bullock found enhanced responsiveness to last longer than 20 seconds in some medusae Facilitated twitches also showed enhanced relaxation Whether the facilitation observed in the nerve net system is myo-neural or neuro-neural is not critically important in terms of the overall integrative properties of the system The nerve net system showed a long refractory period—0 7 sec absolute and 0 1 sec relative in the jellyfish, smooth fusion of response to repeated stimuli never occurred The long refractory period probably belongs to the muscle Ross and Pantin (268) found Mg to block the facilitation They also observed an increase by potassium Bullock (56) confirmed the Mg effect and showed that during the period in which neuromuscular block gradually becomes complete there is a stage when contractions change from general to local responses, these barriers can be broken by repeated stimulation, hence they are apparently due to depressed neuro-neural facilitation Certainly the increased response with increasing frequency is not due to increased contractility (staircase) Bullock presented evidence that facilitation is important in determining strength of contractions in normal rhythmic activity of jellyfish

3 *After-discharge* Pantin (222) observed that as responses build up during repeated stimuli supernumerary responses frequently occur These may represent a sort of after-discharge

4 *"Inhibition"* We mentioned Mayer's blocking of a wave of contraction in a jellyfish ring by pressure Two opposing waves of luminescence in sea fans cancel out when they meet (206), and analogous phenomena have been reported in ctenophores (68) No evidence for periods of depressed excitability, however, has come from the use of paired or repetitive stimulation of anemones or medusae The cancellation of colliding waves may be due to refractoriness of conducting pathways. In a jellyfish ring preparation Kinosita (170) showed that if the

increased, and excitability diminished Opinions are equally divided as to whether tonus is increased or decreased by extirpation of the ganglion Day (81) slit a siphon and observed that a contraction wave was conducted around the cut It seems certain that a direct connection exists from epidermal receptors to underlying muscles in ascidians, but conduction and tonus properties of the muscle need elucidation

Among higher chordates peripheral nerve networks are retained only for coordination of smooth muscle movement, particularly in the gut On the other side of the phylogenetic tree (worms—molluscs—arthropods) peripheral nets are less important in controlling locomotion than on the echinoderm-chordate side

Among flatworms dependence upon central neurons is striking in view of their primitiveness in other respects In free-living flatworms the nerve trunks (2 to 8 in number) run posteriorly from the brain and contain nerve cells which are not grouped into ganglia As long as a portion of one of these nerve cords is left in a piece of a planarian, spontaneous movement and some co-ordinated responses persist (23) Eggers observed (90) this necessity of a bit of remaining cord also among Nemerteans The only example of independent function of the peripheral system is in the planarian proboscis where if the two connections to the central nerve trunks are severed the proboscis autoamputates and shows food-seeking reactions (168)

Annelids, being tubular and containing a movable coelomic fluid, might well be expected to show peripheral co-ordination In the earthworm a subepidermal plexus of branching fibers and scattered nerve cells lies outside the circular muscles, each central ganglion gives rise to three pairs of segmental nerves containing both sensory and motor fibers which connect with the subepidermal plexus Can this peripheral network conduct impulses along the body? Can a strip of earthworm body wall move spontaneously? Is there connection from epidermal sense cells through the peripheral network directly to muscles?

It has long been known that if the ventral nerve cord is cut or is removed from the earthworm for several segments peristaltic waves still pass from one end of the worm to the other Peristaltic transmission occurs if the two pieces of a transected worm are connected by a thread or when a few segments are anesthetized (101, 102) Coonfield (76) found that if part of the nerve cord was removed a wave of mucus secretion failed to pass from normal to denervated segments If the nerve cord is removed from more than three segments and those segments are firmly pinned down so that no pull can be exerted beyond them, no peristaltic wave passes but if less than three segments are denervated and these segments pinned down a wave is conducted (241) Action potentials in the segmental nerves show that each nerve serves three segments, the different nerves serving overlapping fields which are fairly discrete When one nerve is stimulated there is contraction in its own segment and to a less extent in adjacent segments (241) Janzen (151) made a T-shaped cut in the lateral body wall of an earthworm, he observed transmission of a contraction wave "around the corner" if the nerve cord was intact, but not in a strip lacking the nerve cord

Spontaneous contractions have been seen in strips of earthworm body wall

lacking the ventral nerve cord (109, 290, 125, 151, 127) and have been denied (54, 48, 143) An earlier suggestion that spontaneous activity exists in suspended denervated strips but not in non-suspended strips (239) seems not to hold. In segments from which the nerve cord had been removed both muscle layers remained tonically contracted, this was ascribed (143) to a peripheral plexus which normally was inhibited by the central nervous system

Janzen (151) obtained spontaneous periodic contractions in strips which had been narcotized during dissection but not in non narcotized strips, the contractions were usually local but could be propagated, the two muscle layers could contract separately Hatai (127) reported spontaneous activity in dorsal strips from the posterior end of Allobophora but not from the anterior portion and not at all from Perichaeta We have made similar observations on Lumbricus The muscles of segments anterior to and including the clitellum often remained contracted at the edges for hours after excision, this was probably a response of the smooth muscle to injury and not evidence for peripheral ganglia as Von Holst (144) had suggested, these excised segments failed to show spontaneous movements. Post-clitellar strips relaxed but were not spontaneously active Dorsal strips consisting of about twenty segments just in front of the posterior tip, however, showed spontaneous contractions in four out of seven preparations It appears then that posterior regions of the dorsal body wall are capable of spontaneous movement but there is no reason to implicate the subepidermal plexus Janzen's drug experiments are not convincing The body wall muscle may, like the smooth muscle of the mammalian intestine, be capable of some intrinsic movement. Conduction through a sheet of smooth muscle is known and likely occurs in the separate layers of earthworm muscle

Janzen (151) found that tactile stimulation of dorsal body strips elicited contractions, usually local but sometimes propagated, spontaneously active strips showed the best responses We have noted similar effects and obtained contractions both when the tactile stimuli were applied to the muscle side and to the skin side of the strip The responses resemble those of a guinea pig ileum to prodding The body wall muscle may be responding to direct stimulation However, in a few of the preparations from the posterior end a clear-cut response to bright illumination was obtained after dark adaptation It is probable that in the response to illumination and to very light touch there is some direct connection between sense endings and muscle. However, the weakness and the local nature of the response and its virtual absence in anterior segments show that it can hardly be of importance in behavior

The preceding evidence indicates that the subepidermal plexus of the earthworm is not a true nerve net for general conduction, that while there may be connections through it from sense cells to muscle these cannot be of importance in behavior and that there is no evidence that the network plays any part in initiating spontaneous movement of the body wall The peripheral plexus is probably a sensory relay area since there are many more epidermal and muscle sense cells than there are segmental nerve fibers (241) It is possible that among more primitive annelids the connection through the peripheral plexus from sense

organs to muscle was important but that in earthworms the central nerve cord has now taken over complete control Certainly among polychaetes (e g, Arenicola (164)) there is no evidence of any conduction of peristaltic waves except by way of the nerve cord

In gastropod molluscs a subepidermal plexus has been described, particularly in the foot (41, 37) Biedermann (41) found that small pieces of the foot of a slug could show peristaltic movement whereas pieces of the foot of Helix did not show such movement Jordan (159, 161) removed the paired pedal ganglion from Aplysia and noted a marked increase in tonus of the "wings," this was arbitrarily attributed to a release of the peripheral net from inhibition by the pedal ganglion When ganglionic connections to the "wings" of Aplysia were cut (138) and one of the motor nerves was stimulated, there was an immediate contraction of the area served by that nerve followed by a peristaltic wave which spread out from this area This wave was said to be reflex in nature with the peripheral network conducting the impulses Cobb (71) found that excised labial palps of Anodonta show autonomous responses to stimulation by light, touch and chemicals These palps must contain direct connection through the peripheral nervous network from sense organs to muscle In general, molluscan subepidermal networks may relay messages from central ganglia but are incapable of any independent locomotor control There is little doubt that normal co-ordinated locomotion of the foot requires the presence of the pedal ganglia More knowledge of the conduction properties of the muscle is needed before functional importance can be assigned to the peripheral network

The use of a peripheral nerve network in locomotion appears with slight success in the echinoderm-chordate line and with still less success in the annelid-arthropod-mollusc line In the higher groups—vertebrates, polychaetes, cephalopod molluscs, and arthropods there is no evidence of a peripheral network for locomotor control Yet in most of these a nerve network is retained for regulation of movement in the digestive system Central nervous reflexes have become dominant patterns in animal behavior

*Ganglionic Function* Locomotion is under reflex control in annelids, arthropods, and molluscs, but ganglionic reflexes have in no invertebrates been subjected to such complete analysis as have spinal reflexes in mammals Invertebrate ganglia should provide excellent material for future analysis of excitatory and inhibitory states Analyses of behavior in terms of ganglionic reflexes are, however, fairly complete

1 *Annelids* A number of segments of the ventral nerve cord of an annelid, isolated from the body musculature, can conduct a peristaltic wave to the region beyond Normally, however, the wave is reinforced by the segmental reflexes

Collier (74, 75) suspended 20–40 intact segments of earthworms in a saline bath and found no peristalsis if the worms were carefully balanced If, however, a slight stretch (0 1–1 0 gram on the recording lever) or a tactile stimulus was applied, peristalsis resulted Tension and touch elicited peristalsis only if the preparation was normally innervated Moore (207) anesthetized the tactile receptors with $MgCl_2$ and then initiated peristalsis by applying tension

According to Collier (73, 74) a tension induced peristaltic reflex could be inhibited by strong tactile stimulation, e g , by touching the anterior end during a tension wave. Whether the blocking effect of an antiperistaltic wave is due to central inhibition or to refractoriness somewhere in the reflex chain is not clear Maintained tension set up rhythmic contractions at a maximum frequency of one every two seconds, and the response might continue for many minutes. The frequency increased to a maximum as the applied weight was increased If the weight was applied for only 0.5 second, a rhythmic response occurred, a sort of after-discharge which might last as long as 3 minutes A ventral strip containing the nerve cord gave a single contraction, a dorsal strip no contraction, and a piece of intact worm rhythmic contractions in response to stretch (Prosser and Altschul, unpublished)

Gray, Lissmann and Pumphrey (120) made a similar analysis of locomotion in the leech and found that not only tension and contact but also exteroceptive stimuli from the sucker were capable of eliciting peristaltic reflexes The leech nerve cord showed asynchronous spontaneous electrical activity when isolated, and a rhythm corresponding to the contractile rhythm when connected to an active piece of the body wall Peristalsis could be started in an earthworm (119) by electrical polarisation with the anterior end positive, and peristalsis could be inhibited by reverse polarisation

Peristaltic locomotion in the polychaete, Arenicola, is likewise controlled by segmental reflexes, and stimuli at the anterior and posterior ends cause opposite parapodial movements (164) Motion pictures of Nereis in locomotion (118) showed patterns of movement involving clumps of 4–8 parapodia successively Accompanying the wave of parapodial beat the longitudinal muscles on the same side contracted while those on the opposite side relaxed Thus the Nereis crawled in the direction in which the wave traveled over the body and not, as in the earthworm, in the opposite direction

The earthworm peristaltic reflex is segmental or bisegmental in character and involves reciprocal innervation of longitudinal and circular muscles Circular muscles contract while longitudinal muscles relax and the diameter of the animal is reduced Then longitudinals contract, shortening the worm Knowlton and Moore (176) found strychnine sulfate to stimulate the motor system for both sorts of muscle so that under strychnine the worm shortened and also constricted It appears, therefore, that normally one group of motoneurones is inhibited while the antagonists are excited Janzen (151) described correlative reflexes controlling the setae The locomotor reflexes have both homolateral and crossed components

In general, then, the rhythmic contraction waves associated with peristaltic locomotion in annelids require chain reflexes involving tactile or muscle or sucker receptors and antagonistic central arcs What recovery mechanisms are so slow as to determine the frequency of contraction under continuous stimulation is not clear but they may well be in the muscle rather than in the central nervous system

2. *Arthropods* Arthropod locomotion depends upon central conduction and

upon segmental reflexes which show more complex connections than in annelids In larvae of the beetle Lucanus cervus Sasse (271) found peristalsis to depend absolutely upon the integrity of the nerve cord When a segmental nerve was cut that segment was paralysed but conducted a wave to succeeding segments if the cord was intact Mori (211) observed loss of tonus in skeletal muscles of a silkworm if the 2nd and 3rd thoracic or first abdominal ganglia were removed. In several other lepidopteran larvae (noctuids) each segmental ganglion serves its own segment and the one ahead (145)

The reflex control of insect walking and flight and breathing is very precise In 1899 Pompilian (235) noted some automatism of thoracic ganglia for leg movements in Dytiscus Flight can be initiated reflexly by removing the legs from support, i e , tarsal contact inhibits flight (98) Rijlant (259) recorded potentials from leg muscles of scorpions, crayfish, and some beetles He found tonic low potential discharges 30–40/sec during rest and muscle potentials of higher amplitude and frequency during activity When he isolated one or two segments—ganglion and limbs—reciprocal alternate contractions of flexors and extensors in successive segments occurred Pringle (237) made similar observations on the cockroach and noted crossed inhibition

Von Buddenbrock (53) performed a variety of operations upon the nervous system of the walking stick, Dixippus, and concluded that each leg by its motion has a stimulating effect on neighboring legs, excitation passed across a given ganglion and elicited a response from whichever adjacent ganglion was more excitable Bethe (38) upset the normal sequence of leg movement by removing legs from various spiders, crabs, beetles, etc Changes in co-ordination occurred at once Legs which previously alternated now worked together, pedipalps might function in locomotion, swimming was carried out by other legs than normally Holding a leg was not equivalent to removing it The changes occurred too fast for learning and must represent pre-existing pathways and an adaptive property of the ganglia

Ten Cate (302) performed amputations upon the appendages of the cricket, Locusta viridissima, and obtained alterations in co-ordination if he removed a foot only He concluded that the normal sequence and rhythm is set up reflexly, and is not a pre-established central pattern Not only can a change in leg rhythm be brought about by amputation but similar changes occur with normal changes in gait The mantis leg order, for example, (262) when quietly walking is L 3, L 2, R 3, R 2, when excited and walking fast the rhythm is L 2 + R 2, L 3 + R 3, when climbing L 3 + R 1, L 2 + R 3, L 1 + R 2 In some insects the order of leg movement depends much on the surface, i e , the order is determined by proprioceptive reflexes (146)

Prosser (242) recorded action potentials from various segmental nerves and from the central nerve cord of the crayfish By stimulating different appendages and making appropriate cuts he obtained a functional map of the nerve cord. The available connections are multiple, which ones are used in a given behavior pattern must depend upon the strength of stimulus and upon the ease of passage through a particular reflex arc

Locomotor rhythms in insects and crustacea, as in annelids, are reflex in nature and a variety of patterns of locomotion may be used depending upon external requirements

3 *Molluscs* In gastropod and pelecypod molluscs locomotor reflexes are mediated by three pairs of ganglia, cerebral, pedal and visceral

Pavlov in 1885 (233) experimented with the mussel, Anodonta Spontaneous activity of the foot stopped when the visceral ganglion was removed but tonus could still be maintained in the adductor muscles after they were denervated The posterior adductor muscle received motor (exciting) impulses from the visceral ganglion, the anterior adductor from the cerebral ganglion, both ad ductors received inhibitory fibers from the cerebral ganglion In the razorshell clam, Ensis (85) local stimulation of the foot failed to elicit general responses if the sensory connections in the cerebral ganglia were eliminated. In Mytilus (331) all of the ganglia act somewhat independently, e g , if the cerebral ganglion is removed the foot still spins and creeps, due to pedal control, if the visceral ganglion is removed opening and closing reactions to changes in freshness of water are lost. All of these observations argue for fairly restricted control of local areas by specific ganglia in pelecypods

In gastropods the pedal ganglion gives off a series of nerves which appear to discharge successively to elicit peristalsis along the foot (41) Co-ordination in the large marine gastropod, Aplysia, has been extensively studied When the pedal ganglion was removed (159, 161) the foot showed more resistance to stretch (tonus) and contractions occurred, particularly at the foot margins When the cerebral ganglion was removed there was increased locomotor activity and in creased excitability of the "wings" (foot) It has been suggested, therefore, that the cerebral ganglion inhibits locomotor functions of the pedal ganglion and that the pedal ganglion inhibits tonic mechanisms in the foot (161) Ten Cate (297) stimulated the central ends of nerves from the "wings" and found that each contains sensory fibers which reflexly through the pedal ganglion elicit contractions in certain areas of the other side of the foot The cerebral ganglion was not necessary for this response If the interpedal commissure was cut, only the homolateral responses persisted In Helix, foot potentials indicated that the cerebral ganglion inhibits motor cells of the pedal ganglion (132)

Ganglionic reflexes control the muscles and chromatophores of the mantle and fin of cephalopods The mantle nerve goes from the brain mass to the mantle or stellate ganglion and from this the stellar nerves pass to the mantle Fröhlich (107, 108) found that the mantle ganglia maintained tonus of the mus cles and chromatophores of the mantle Stimulation of the mantle nerve at low intensity inhibited the stellate ganglion, at higher intensities excited it. Gan glionic delay of 10 msec occurred and summation was indicated. Fatigue of a stellar nerve did not affect the mantle nerve. When the mantle nerve was cut a weak reflex response could be elicited through the stellate ganglion alone On the contrary Bozler (51) maintained that the stellate ganglion is a motor center which cannot mediate reflexes of the mantle but Ten Cate (299) confirmed Fröh lich and showed that reflex contraction could be elicited over either of two path

ways, via the brain mass (traversing the stellate ganglion for both afferent and efferent fibers), or via the stellate ganglion alone Electrical studies on these widely separate ganglia should be possible

Molluscs, in general, have well developed local reflex centers which may show some interaction but which have relatively restricted control of particular sensory and motor areas In this respect molluscs are even more highly specialized than the segmental arthropods

*Spontaneous Ganglionic Activity* Locomotion in annelids, arthropods and molluscs is reflex in nature Isolated portions of the central nervous system in annelids and arthropods show continuous spontaneous electrical activity Among molluscs such activity has been seen only in the slug, Ariolimax (59) This activity does not in most cases represent summated rhythmic discharges of many neurones as seems very largely to be the case in the vertebrate brain but is asynchronous (240, 244) In most ganglionic chains each neurone is independent of adjacent ones although a given neurone may discharge more or less rhythmically, different neurones having different frequencies The spontaneous impulses find their way out to body muscles and are tonic in character (152), incoming sensory messages are superimposed upon the spontaneous background

Certain invertebrate ganglia, however, do show synchronized rhythms Bullock (59) obtained slow waves upon which spikes were superimposed from central ganglia of grasshopper and Limulus Breathing rhythms appear to be intrinsic in respiratory centers, at least in many insects Lesions to specific abdominal ganglia have been shown to stop the breathing rhythm In Schistocercus, a locust, Fraenkel (99) found breathing centers in abdominal segments 5 and 8, whereas centers for closing the stigmata were in the thorax In Limulus all or part of the chain of abdominal ganglia, when entirely separated from the rest of the nervous system, can maintain normal movements of the gills (150) Strong support of the neurogenic origin of breathing rhythms in arthropods came with the observation of waves of nerve impulses in isolated nerve cords at rates corresponding to breathing rates (Dytiscus, 1, cockroach 244) These breathing centers are subject to much sensory regulation In certain insects during flight an increase in breathing amplitude and opening of stigmata and a decrease in breathing rate were found (99)

Another summated rhythm in an arthropod ganglion is the synchronization observed by Adrian (2) in the optic lobes of the brain of Dytiscus When the eyes were illuminated a rhythm at 20–40 waves per second built up in a synchronization of most of the neurones in the lobe In darkness this "light" rhythm stopped but after some hours a "dark" rhythm at 7–10 waves/sec appeared

Another type of summated rhythm is found in neurogenic hearts The pacemaker region of the heart of most crustaceans, insects and probably annelids is a ganglion or group of nerve cells (246) As with the asynchronous central ganglion it has been possible to analyse the heart rhythms in terms of single neurone activity In the Limulus heart ganglion each large pacemaker cell

appears to give rise to a slow wave of negativity for each heart beat and then these and small motor cells nearby discharge a volley of impulses (131, 247) These impulses start at a high frequency and taper off toward the end of the volley

Other invertebrate ganglia are capable of spontaneous rhythms The so called sense organs at the margin of a medusa initiate the pulsations of the jelly fish (266, 199, 49) Wells (317) described a complicated rhythmic activity of the esophagus of Arenicola, the origin of the wave of nervous activity apparently being in part of the stomatogastric nervous system

Many invertebrate ganglia, then, show spontaneous activity, this may be asynchronous or synchronized Single neurone analyses can be made more easily than with vertebrate nerve centers, and some of these ganglionic preparations are useful for elucidating cellular mechanisms of spontaneous rhythms

*Cephalic Dominance* The next step in complexity of nervous function beyond local or segmental reflexes is increasing importance of particular centers near the anterior end of the animal This importance seems to depend first upon the concentration of sense organs at the front end of the animal and second upon co-ordinative functions of the "brain" ganglia

1 *Flatworms* The sensory basis for cephalic dominance is illustrated in the free-living flatworms where the brain is associated with eyes and other sense organs A number of papers deal with the effects of removal of the brain from flatworms In the polyclad Thysanozoon the brain is needed for spontaneous locomotion (183), and in the polyclad Yungia immediately after removal of the brain or head the brainless worm or remaining posterior part is inactive and less sensitive than normally but can be stimulated mechanically or chemically to perform swimming movements (208) In Yungia some hours after the operation spontaneous and co-ordinated but undirected movement occurs Olmsted (216) found in some marine polyclads that swimming stopped but ciliary action persisted if the brain were removed or split The rippling movement (ataxic locomotion) continued, but extension, placing and release (ditaxic locomotion) and peristalsis stopped since these required conduction from the brain back over lateral cords Righting appeared to require the brain in the polyclad Planocera (209), but not in the triclad Planaria (183) The greater importance of the brain in polyclads than in triclads may be associated with the distribution of ganglion cells In Planaria locomotion and reflex extension of the pharynx and swallowing continue after removal of the head but normal behavior to food reappears only after the brain regenerates (23)

In cestodes after the lateral cords are cut a wave of contraction progresses backward across the cut, being conducted reflexly by tension applied behind the cut (254) Friedrich (103) found that cutting one lateral cord resulted in circus movements in those species of nemerteans in which transverse connections between lateral cords were incomplete, but not where the cords were connected In summary, the flatworm brain relays sensory messages and co-ordinates motor responses, there being no other ganglia, its directive function is, therefore very important.

2 *Annelids* An earthworm has sensory cells of various types—tactile, chemical and light receptors, scattered throughout the epidermis, but most concentrated in the prostomium and anterior segments Only the sense cells which are sensitive to dryness seem restricted to the prostomium (232) Nereis, on the other hand, has fewer epidermal receptors, its effective chemoreceptors are on palps and tentacles and instead of scattered photosensitive cells it has several pairs of eyes on its head After the brain is removed from an earthworm the anterior segments are lifted upward, it crawls normally, appears restless and active, it can right itself, can copulate, eat, and burrows in half an hour as compared to a normal time of one to two minutes (198, 239) After the brain is removed from a Nereis it no longer feeds, does not burrow, is over-active and has lost its light sensitivity and most of its chemical sensitivity (183, 198) When the sub esophageal ganglion is removed from either species there is no burrowing and the worms are very quiet The brain, then, is a sensory center and it normally has an inhibitory or restraining control over the motor centers in the subesophageal ganglion

An example of integrative cephalic dominance which has a simple morphological basis is found in the responses of an earthworm to light An earthworm normally responds negatively to lateral illumination When the brain is removed the direction of its response is reversed (134) If the ventral cord is cut a few segments behind the subesophageal ganglion the anterior tip turns away from the light while the region behind the cut tends to bend toward the light If one esophageal commissure is cut the worm tends to circus toward the normal side and when illuminated from the intact side the worm turns toward the light but when illuminated from the operated side turns away from the light (238, 148) Apparently the scattered photoreceptors set up a homolateral reflex in the ventral ganglia, a positive response to light, the tracts from the prostomium and first two segments, however, cross in the brain and cause a negative response The brain normally dominates but at very low light intensities a positive response may occur

The brains of some polychaete worms are complex and have lobes corresponding to the specialized forebrain centers of arthropods (123) These lobes (corpora pedunculata) are best developed in free-swimming polychaetes which have cuticular eyes, they do not occur among oligochaetes It has been shown that Nereis (77) can be taught simple associations, Lumbricus (334, 130) and Lumbriculus (252a) can be taught even when the brain is not present An investigation of integrative functions of the brain of Nereis should be very interesting in view of the complexity of its brain

3 *Arthropods* There is much variation in complexity of the brain among different groups of arthropods In general there are three regions the protocerebrum, the deutocerebrum and the tritocerebrum The bulk of the lateral portions of the protocerebrum is devoted to vision centers which are directly connected to the eyes, the middle and anterior portions contain the association areas, the protocerebral bridge, the central body, and the large cellular corpora pedunculata There are no corpora pedunculata among some lower crustacea The

deutocerebrum usually lies ventro-anteriorly and may contain large antennal centers as well as "olfactory" lobes The tritocerebrum lies behind and customarily gives rise to certain nerves going to mouthparts as well as to the stomatogastric nerves, the tritocerebrum is continuous with the circumesophageal commissures.

In view of the complex instinctive behavior and limited but real modifiability of behavior in arthropods, especially among insects, an understanding of the function of different parts of the brain should be useful Hanström (123) has compared the relative portions of the brain occupied by different regions in various groups as follows

| | | | |
|---|---|---|---|
| Vision centers | Arachnoidea and Myriapoda | epithelial photoreceptors and ocelli | 0 3– 2 8% |
| Vision centers | Crustacea and Insecta | rudimentary compound eyes | 2 9– 9 9% |
| Vision centers | Crustacea and Insecta | higher compound eyes | 33 0–80 0% |
| Olfactory centers | Crustacea | | 10 0–30 0% |
| Olfactory centers | day flying insects | | 1 3– 4 4% |
| Olfactory centers | one night flying insect | | 13 4% |
| Olfactory centers | worker ant (Formica) | | 18 1% |
| Central body | Arachnoidea | | 1 6–15% |
| Central body | Crustacea | | 0 2– 0 5% |
| Central body | Insecta | | 0 2– 0 6% |
| Corpora pedunculata | Limulus | | 78 7% |
| Corpora pedunculata | Arachnoidea | | 2 0–49 0% |
| Corpora pedunculata | decapod Crustacea | | 10 0–30 0% |
| Corpora pedunculata | isopod Crustacea | | 0 0% |
| Corpora pedunculata | Odonata Lepidoptera Diptera | | 2 0– 9 6% |
| Corpora pedunculata | worker ant (Formica) | | 40 4% |

Since the days of Aristotle, the observation of behavior of arthropods from which parts of the body have been removed has been a favorite pastime A complete survey of papers up to the year 1930 dealing with the effects of operations on the nervous system is given by Ten Cate (301)

The following is a list of the most important animals on which operative studies on locomotion have been made, with references to the papers

*Crustacea* Squilla—35, Palaemon—82, 86, Homarus—196, Astacus—35, 124, 160, 287, 314, various crabs—34, 82, 86, 133, 234, 287, 312, Cancer—160, Maja—287, Eupagurus 300, Portunus—133, Oniscus—287

*Xyphosura* and *Arachnida* Limulus—150, various spiders—260

*Myriapoda* Lithobius—67, 153, 287, Julus—70, 287, Scolopendra—62

*Insecta* Dytiscus—96, 235, Hydrophilus—35, Carabus—19, 111, 296, Lucanus larvae—271, Melanoplus—95, Mantis—262, Ranatra—140, Periplaneta—20, 21, 296, dragon fly nymphs (Aeschna larvae)—22, 313, (Clocon and Agrion larvae)—3, Apis—35; Lymantria larva—177

Similarity of results indicates that further investigations by these gross methods would not be fruitful

Removal of any part of the brain which receives tracts from any sense organ is equivalent to removing that sense organ Immediately after removing a sense organ or injuring the brain, there may be a shock reaction of general incoordination Thereafter, specific behavioral deficiencies appear Most arthropods can eventually compensate for the removal of one eye or of an antenna, whereas injury to the brain leaves a more permanent behavior deficiency If both circumpharyngeal commissures are transected, reflexes of the head such as antennary movements remain, otherwise the effect resembles that of total brain removal Spontaneous directed locomotion ceases, although locomotion is possible under stimulation Locomotion is less impaired by brain removal in some species (Carcinus and Eupagurus) than in others (Astacus) Mouth-parts may move, but usually there is no co-ordinated feeding Some animals can chew but cannot swallow after brain removal Righting and leg reflexes are unimpaired Usually there is extensive random activity of legs

When one circumesophageal commissure is cut, or one lateral half of the brain destroyed, the arthropod circuses toward the intact side Legs on the operated side tend to be flexed and low in tonus so that the animal leans toward this side

There are many reports that removal of the subesophageal ganglion, or cutting behind this ganglion stops spontaneous activity Local segmental reflexes, however, persist The subesophageal ganglion is apparently the principal motor center, but the other thoracic and abdominal ganglia are capable of carrying out reflexes of locomotion, autotomy, and so forth, these are normally regulated by the subesophageal ganglion

The increased leg activity after brain removal suggests the release of the ventral motor centers from inhibition by the brain Similarly, when the brain was removed from dragon-fly larvae (197, 84), there was an increase in breathing frequency (although recovery of the normal rhythm has been reported (313)), while when the subesophageal ganglion was removed, the breathing frequency (determined by posterior ganglia), was decreased Death-feinting is a function of the nervous system as a whole rather than of any particular ganglion, in some insects and in spiders it is relatively unaffected by brain removal In others, as the spider Celaenia (260), there is no death-feinting after brain removal In Ranatra, the death-feinting reaction is shortened after brain removal (140), the posterior half of the body with nerve-cord cut comes out of a feint sooner than the part of the body containing the brain The most convincing evidence for inhibitory action of the brain on the ventral ganglia comes from electrical stimulation Jordan (160) stopped circus movement in a crab by a weak electrical stimulation of a transected circumesophageal commissure *Celesia (65)* caused a flexion of the abdomen of Palinurus and Homarus by tetanic stimulation of the abdominal nerve-cord, if during this stimulation the brain was also stimulated, the abdominal response was diminished Caselli (64), also working on Palinurus, stopped the responses of tail and cloaca to abdominal stimulation by simultaneous stimulation of the circumesophageal commissures

The interaction between the brain and subesophageal ganglia is clearly shown by the work of Roeder on the praying mantis (262) When one side of the protocerebrum was removed the legs lost tone on the operated side, the legs on the operated side were more active and the leg sequence changed, circusing was to the normal side When both protocerebral lobes were destroyed, leg tonus was lost on both sides, there was locomotor restlessness, the mantis walked straight ahead, did not avoid objects, and showed no head motility or backward walking When the protocerebrum was split there was decreased leg movement, high neck and prothoracic tonus and active visual responses (following moving objects) When the subesophageal ganglion was removed there was no locomotion except to strong stimulation Roeder concluded that the subesophageal ganglion excites locomotor activity in thoracic ganglia, that the protocerebral ganglion has inhibitory centers controlling the irritability of the subesophageal locomotor center and excitatory centers maintaining tonus and activity in neck and prothoracic muscles, these protocerebral centers are homolateral, are inhibited contralaterally by each other and strengthened by homolateral sensory impulses Roeder noted that sometimes in mating, after clasping, the female eats the male, head first, and copulation continues actively Experimentally he found (261) that if the brain was removed there was no sexual activity, but if the subesophageal ganglion was removed the male made copulatory movements and the female would receive the male. The copulatory center is in the last abdominal ganglion and this is normally inhibited by the subesophageal ganglion Thus the eating of the head (and subesophageal ganglion) by the female actually releases copulatory activity!

Some arthropods exhibit very complex behavior, some of which may require the presence of the brain A brainless hermit crab for example (300) will not re-enter its shell unless it is helped by having the telson pushed in, the hermit crab will grasp a new shell but fails to investigate it as a possible dwelling-place. In some arthropods (female Bombyx—201, and Carcinus—35), mating can occur in brainless individuals, whereas in others (various butterflies,—177), it does not. There is ample evidence that some insects have capacity for learning Von Frisch (105), for example, taught honey-bees to come to glass dishes set over certain colors and patterns Experiments concerning the possible relation of parts of the brain to learning should be very profitable

The function of the specific lobes of the brain can be partly inferred from their connections as shown in the table from Hanström given above Bethe (36) found that removal of the globuli (the cellular portion of the corpora pedunculata) in Carcinus caused the loss of certain responses to light, and of sidewise locomotion, he stated that the globuli are inhibitory centers of the brain In the myriapod Lithobius (153) the protocerebrum inhibits the ventral motor centers and controls the sense of direction Roeder's results are in agreement (262) Ewing (95) on the other hand, stated that the legs of a grasshopper went into constant motion when only the posterior part of the brain was removed.

In summary, then, the brains of arthropods not only are connected with the most important sense organs of the body, but they also exert important in-

tegrative, particularly inhibitory, control of the ventral motor centers It should be profitable to investigate further localization of function within the arthropod brain and the mechanisms of interaction between the brain and ventral ganglia, and between the subesophageal and other ventral ganglia

4 *Cephalopods* Cephalic dominance has developed in a somewhat different manner in cephalopod molluscs The brain and some thoracic ganglia of arthropods represent fusion of several ganglia The cephalopod brain-mass represents fusion of paired cerebral, pedal, and visceral ganglia, and completely surrounds the esophagus (309, 270) Paul Bert in 1867 (33) was the first to show that much of the supraesophageal portion of the brain-mass was, like the association areas in the mammalian cerebral cortex, electrically inexcitable with respect to behavior He also showed that a respiratory center exists in the subesophageal portion of the brain-mass Von Uexküll (309) divided this respiratory center into regions for inspiration and expiration He and other early workers (172) also found a center in the subesophageal portion which regulates chromatophores The subesophageal portion contains other local centers (270) the brachial and pedal ganglia controlling arms and tentacles, the pedal ganglion controlling funnel and eye-muscles, the pallio-visceral ganglion controlling mantle, fins and viscera In Octopus a center for pupillary closure is found in the subesophageal portion (316)

The supraesophageal portion (270) contains (1) Higher motor centers in the circumesophageal region—lobus basalis anterior, posterior and lateralis, lobus pedunculi, stimulation of these lobes elicits movements of large groups of muscles, and unilateral extirpation results in circus movements which may be almost continuous (2) Primary sensory centers such as the olfactory lobes and the optic lobes in the optic stalk, stimulation of the latter may result in chromatophore expansion and some mantle and fin movement (3) The dorsally located verticalis complex of three lobes, electrical stimulation of which results in no motor responses After removal of the entire verticalis complex Sepia can see, swim, steer properly, capture and eat prawns, etc However, when a prawn which it was hunting went around a corner the operated Sepia failed to follow it out of sight as normal animals did (270) Removal of part of the verticalis complex from Octopus hampered its ability to escape from a cage and from a pan of shallow water (61) By conditioning experiments specimens of Octopus (304) were shown to be able to discriminate between square and circle while Sepia (270) was trained not to eject its tentacles at a prawn behind a glass bearing a white circle but to continue to shoot them at prawns not accompanied by glass and circle Sanders and Young (270) conditioned three individuals not to eject their tentacles at a prawn behind a glass plate, "memory" of this lasted eighteen hours, the learning of this association occurred after about the same number of trials with or without the verticalis complex

It appears that the verticalis complex is an association area but that conditioning of other centers can occur

*Neuromuscular Integration* Among vertebrates integrative functions are

largely a property of the central nervous system, gradation in control of movement is carried out largely in the ventral columns of the spinal cord. Among most invertebrates there are numerous ganglia each of which can regulate locomotor reflexes Among many invertebrates much control of gradation of movement is at neuromuscular junctions rather than centrally A crab "thinks in it claws"

Early histological studies (40, 139, 191) showed (1) that whole muscles in many crustaceans and insects are innervated by only two (or a few) axons and (2) that each muscle fiber has multiple innervation, i.e., each muscle fiber receives branches from two or more nerve fibers, often from all the motor fibers to the muscle. Triple innervation of claw muscles in the crayfish was described by Van Harreveld and Wiersma (307) and even quintuple innervation of a muscle of the leg of Panulirus (308) Pringle (230) described double innervation of leg muscles of a cockroach It is likely that multiple innervation of fibers and innervation of all the fibers of a muscle by the same axon is a general rule among higher crustacea and insects

Stimulation of claw nerves of Crustacea early revealed that at low intensities of repetitive stimuli the adductor muscle contracted while at higher intensities the same muscle relaxed (was inhibited) and the abductor contracted (40, 106, 139, 311) It appeared that the peripheral inhibition was associated with double innervation Keith Lucas (184, 185) showed that in the claws of lobsters and crawfish a slow low level contraction of the adductor resulted from a weak long-duration stimulus and a rapid strong twitch from a strong brief shock. On the basis of thresholds, effective stimulus durations and separate intensity-duration curves he concluded that there were two *excitatory* systems to the same muscle, one fast and one slow Paired stimuli resulted in summation of response in each system

A further complication in crustacean neuromuscular conduction is the fact that sometimes single strong shocks applied to a crustacean nerve elicit repetitive discharges in the nerve (24) This repetitive response depends upon the calcium in the medium bathing the nerve (166) Barnes suggested (24) that gradation in response depends on the frequency and duration of the repetitive discharge. This might explain the following observations of Blaschko, Cattell and Kahn (43) on the claws of Homarus, Cancer, and Maia The tension of the contraction increased gradually as the frequency of repetitive stimuli increased, more muscle fibers coming in with increasing frequency Normally the slow closing response occurred at 2 to 10 per sec and the fast response at 50 to 100/sec If the slow type response was being elicited and a single extra or strong shock interposed, suddenly the contraction changed to the fast closing type and continued at this level even though stimulation was at the original low level

Crustacean neuromuscular junctions, then, behave very differently from those of vertebrates Segaar (277) suggested that the effect of a nerve depends on the tonic state of the muscle so that some fibers can evoke either excitation or inhibition This suggestion lacks experimental verification Segaar also claimed that stimulation of the brain elicits opposite responses from stimulation of a

peripheral nerve (133, 277) Tonner (305) claimed to find a subepidermal network which maintained tonus in the muscles and which was influenced by impulses from the nerve centers His views have found no support

Pantin in a series of papers (218, 219, 224, 225, 226) analysed the effects of frequency, duration and intensity of stimuli to the nerve upon responses of walking leg muscles (largely flexors) in Maia and Carcinus At low intensities there was little or no response to a single shock, a greater response after several shocks The response (tetanus) increased with increasing frequency or increasing intensity With intensities just above threshold most units were brought into action at 60–100 shocks per second and a maximum response occurred at 300 per second Thus the response depended upon the number of impulses arriving in the muscle in a given time If the frequency or intensity were increased abruptly by 10–20 times threshold the rate and height of contraction increased, i e , a quick response was brought in This Blaschko, Cattell and Kahn effect could also be obtained by stretching the muscle during repetitive low intensity stimulation When the quick response was fatigued the slow response was also Pantin contended that the bringing in of the quick response was due to repetitive nerve impulses set up by the interposed stronger stimulus This would make for economy as a few extra impulses coming from the nervous system on a low frequency background could change the response from the slow to the fast type If brief tetani were given at increasing intensities and both the flexor (adductor) and extensor (abductor) muscles studied there was first a contraction of the extensor, then at a higher intensity the threshold of the flexor was reached but the extensor contraction diminished and at still higher intensities the flexor was inhibited Thus Pantin arranged the four fibers in order of increasing threshold as follows excitor of extensor, inhibitor of extensor, and excitor of flexor and finally inhibitor of flexor He also was able to separate the four fibers by strength-duration curves The inhibition was less effective against high frequency stimulation (fast contraction) than against low frequency stimulation Pantin concluded that the response in the crustacean neuromuscular system of the walking leg depends upon facilitation, more muscle fibers responding as more impulses arrive at the muscle, that the fast response is the result of high frequency impulses, that the Blaschko, Cattell and Kahn effect is due to repetitive firing and that inhibition results from impulses in the second or higher threshold axon innervating a muscle In the crusher claw Pantin obtained evidence for a double excitor system controlling the slow and fast responses Pantin's views were supported by Katz (166) who demonstrated summation of both electrical and mechanical responses of the Carcinus walking leg He found the largest increase in number of responding fibers at between 60 and 120 shocks per second and noted repetitive nerve responses to a constant current

Electrical responses have frequently been recorded in crustacean muscles DuBuy (87) used tiny knob electrodes for recording and detected a fast nerve potential followed by a slower muscle response He found a negative excitatory wave and a positive inhibitory wave and stated that the muscle would respond to adrenalin without an action potential The observation of positivity in

inhibition has not been repeated Serkoff (278) stated that inhibitory impulses to a resting muscle did not alone set up any electrical phenomena but might or might not reduce the height of the potential in a contracting muscle Sommer (286) supported Pantin's views that the response is a function of the frequency of stimulation, in Astacus he found that an increase in frequency by ten times increased the amplitude of the potential about three times, stimulation of the inhibitor nerve and of the excitor simultaneously diminished the muscle potential while stimulation of the inhibitor nerve alone resulted in no electrical wave in the muscle

From 1933 to the present Wiersma and his collaborators, particularly Van Harreveld, have published a series of papers which constitute the most extensive and definitive work on this subject Wiersma's chief technical contribution is the isolation and identification of function of single motor nerve fibers It had been indicated earlier and definitely shown by Knowlton and Campbell (174) (also Knowlton, 175) that the claw nerve of the lobster could be split into bundles. One containing the larger motor fiber caused contraction of the adductor and inhibition of the abductor The smaller fibers on the other hand had the reverse effect. Wiersma and his associates have traced the course of individual fibers by methylene blue staining and have identified their action by stimulating the separate nerve fibers and recording contractions and muscle action potentials Their work was summarized at length in 1941 (321) and only a brief description of it can be given here.

The variation in pattern of nerve fiber distribution among different species and in different muscles of one species is remarkable. Some muscles receive one excitatory and one inhibitory fiber, in these muscles the height of contraction depends upon facilitation, the walking leg muscles studied by Pantin are examples Some muscles receive two excitatory and one inhibitory fiber In these the largest fiber, the "fast" one, causes a contraction of short latency, rapid shortening, and high tension development, the fast fiber may elicit an action potential which appears with the first of a train of impulses and shows slight facilitation for the first few spikes after which the contraction begins (306) Stimulation of the second or intermediate fiber, the "slow" one, causes a contraction which builds up very slowly so that there is an apparent long latency and a low tension, the muscle action potentials are smaller than from the "fast" fiber stimulation, they may not appear until after several stimuli and they show much facilitation Stimulation of the third and smallest fiber causes inhibition of contraction, reduces the height of contraction and in some muscles reduces the height of the action potential, inhibition is more effective against the slow than against the fast contraction During complete inhibition no contraction occurs, but facilitation continues to be built up in the slow system as shown on release from inhibition The distinction between "fast" and "slow" responses is quantitative rather than qualitative At low frequencies (40–50/sec) the difference between the two fibers may be very slight or the larger fiber may even give a smaller contraction but at high frequencies (over 100/sec) the fast contraction is much greater (326) Thus the optimum frequency in the slow fibers is lower

than in the fast fibers The difference between the fast and slow contraction of the same muscle becomes less in the species series Cambarus > Randallia = Blepharipoda > Cancer so that in Cancer there is little difference between the two except latency (326) The flexor of the carpopodite of Panulirus has five axons, 4 excitors of different rates and one inhibitor (308) The amount of facilitation required to elicit a contraction varies with the species, e g , in the crayfish the fast system gives a detectable response to one shock, the slow system requires more than one while in Blepharipoda repetitive stimuli are required for both systems (327) The ratio of optimum inhibitory to excitatory frequencies is fairly constant for any given system (323) For example, in the slow bender of Pachygrapsus three excitor impulses are suppressed by one inhibitor, in the slow closer of Cambarus one excitor requires three inhibitor impulses, while the fast closers of these species respond to single excitor impulses and are uninhibitable

In several instances one nerve axon serves two muscles (320) For example in the crayfish claw (Cambarus clarkii) the dactylopodite (pincer) abductor has one excitor which it shares with the extensor of the protopodite while the latter muscle shares its inhibitor with the adductor of the dactylopodite (307, 329). In Cancer (322, 324) the abductor of the claw has two inhibitor fibers, one its "true" inhibitor reduces muscle potentials if stimulated just before the excitor, the other its "common" inhibitor is shared with the adductor of the same segment and is without effect on the potential of the abductor but reduces its contraction and slightly reduces the potential of both fast and slow systems of the adductor Often reciprocal innervation does not apply for a pair of antagonists and at least one muscle receives two inhibitors (322) Regenerated claws may have more motor axons than normal claws (306, 25, 320)

When both excitor and inhibitor fibers are being stimulated repetitively, the inhibitor fiber may cause diminution of both contraction and action potential if its impulses arrive less than 10 msec before the excitor impulses ("supplementary" inhibition), or the inhibitor may cause reduction of contraction only, if its impulses precede the excitor impulses by longer times ("simple" inhibition) (191a)

All evidence indicates that every fiber in a muscle receives a branch from every axon Each fiber, then, must contain several excitation systems Keighley and Wiersma (167) showed that the slow system is more efficient than the fast one in that its ratio of heat to mechanical work is lower However, chemical changes are of the same order with the two types of contraction (32) It is possible to produce fatigue and facilitation among the different excitor systems separately (328), one inhibitor can suppress two or more contraction types How so many types of response can be elicited in the same muscle fiber is a major problem for muscle physiology Wiersma (321) presented the following scheme for each excitor system nerve impulse → transmission process → muscle potential → transmission process → contraction He suggested that facilitation can occur at either transmission process and that the contraction system is common to several kinds of nerve impulse, "simple" inhibition blocks between

the muscle potential and the contraction, whereas "supplementary" inhibition blocks between the nerve impulse and the muscle potential

In general, crustacean muscle differs from vertebrate striated muscle in showing multiple innervation, the same axon serves many muscle fibers so that conduction throughout the muscle can be nervous, some muscle fibers receive axons which elicit contractions of different speeds and tensions, facilitation is essential for most contractions, more for the slow than the fast ones, a nerve fiber may discharge repetitively to a single stimulus, there are specific inhibitory nerve fibers Regulation of the height and speed of a contraction is brought about by (1) variation in frequency (facilitation), (2) action of fast and slow nerve fibers, (3) inhibitor nerve fibers, and (4) by continuation of a high level response at low level stimulation after a few high frequency impulses

Multiple innervation of muscles exists not only in Crustacea but also in insects (191) Each of the leg muscles of a cockroach receives two nerve fibers (237) One of these causes a tonic contraction and discharges when attached normally to the nervous system at 30/sec while the other gives bursts of large spikes at 75/sec and causes a quick high level contraction (236) The slow system shows much facilitation, the fast system little or none. No inhibitory fibers were found, but antagonism between extensors and flexors involving central inhibition in unisegmental reflexes was shown by appropriate sensory stimulation of intact preparations (237) Friedrich (104) obtained functional evidence of multiple innervation in the control of peripheral inhibition of the leg muscles in Dixippus

In the clam Mya arenaria, the adductor muscles and mantle retractor muscles receive two types of nerve fiber (249) One fast type elicits a rapid contraction with large action potential, the other type causes a prolonged tonic type of contraction with a low level electrical response Such a system is efficient when a low level contraction must be maintained and occasional quick contractions superimposed The retractor of the buccal mass of Helix appears not to be doubly innervated (253) In Pecten the adductor muscle is composed of a striated portion used for rapid swimming movements and a smooth portion used for prolonged maintained contraction (26) During swimming the smooth muscle is inhibited and part of this inhibition is probably peripheral (30)

We have found *no information as to whether multiple innervation exists among* annelids or echinoderms

Many invertebrates, then, carry out much regulation of movement by peripheral neuromuscular mechanisms, regulation which in the vertebrates is carried out in the spinal cord

*Chemical Agents* The nature of synaptic transmission in invertebrates is virtually unknown. No analyses of ganglionic potentials have been made although many measurements of synaptic delay are available.

In view of the importance of acetylcholine in sympathetic ganglia, at certain neuromuscular junctions and at numerous other nerve endings in vertebrates it is reasonable to inquire into the origin of cholinergic systems The effects of acetylcholine and adrenalin upon invertebrate hearts, chromatophores, and

visceral musculature are not pertinent to this review It should be said, however, that evidence from a few perfusion experiments and from the effects of drugs upon invertebrate hearts indicates that acetylcholine may have much to do with cardiac regulation in many animals Acetylcholine accelerates neurogenic hearts (most arthropods and probably most annelids) and inhibits myogenic hearts (molluscs and vertebrates) (246)

Information concerning cholinergic systems can be obtained from (1) measurements of cholinesterase content of nervous and muscular tissues, (2) measurements of acetylcholine (Ach) in these tissues, and (3) sensitivity to acetylcholine and to its potentiating and antagonizing drugs Available data on cholinesterase content of invertebrate tissues and a few representative vertebrate tissues are summarized in table 3 The data presented in this table are not all comparable because of differences in method and in laboratory The highest concentrations of cholinesterase reported in vertebrate tissues are in electric organs of fish, the highest in mammalian nervous tissue are in sympathetic ganglia (except dog lenticular nucleus) A ganglion of the squid is the highest invertebrate tissue reported although the lobster ganglia, honeybee brain and Limulus heart ganglion appear to be rich in cholinesterase Cholinesterase is found in nervous and muscular tissues in all of the groups tested down to coelenterates and ctenophores Among coelenterates Bullock and Nachmansohn (60) found the enzyme in the hydrozoans and anthozoans Tubularia, Metridium and Sagartia but found none of it in any regions of the medusae Cyanea and Aurelia, they found none in the ctenophore Mnemiopsis They also found none in the sponge Grantia or in Paramecium although adequate masses of tissue were used Bacq (10) also found none in Rhizostoma and sponges although he found much in the muscle of Holothuria The concentration in flatworm mixed muscle and nervous and other tissue is strikingly high

The activity of invertebrate extracts towards other substrates than ACh has been examined in the honeybee brain where there appeared to be proportionately very much more "true" than "pseudo"-cholinesterase than in mammalian sympathetic ganglia

Determinations of acetylcholine content are less dependable than determinations of the esterase because of hydrolysis and necessity for quick inactivation of the esterase before assay Further, there is uncertainty as to how acetylcholine may be "bound" in tissues Such results as are available are given in table 4 Crustacean ganglia compare favorably with mammalian sympathetic ganglia and some insect ganglia appear to be even higher in acetylcholine content Octopus ganglia contain the highest concentration yet observed (except possibly for some insects) Bacq and Mazza (17) purified and chemically identified this acetylcholine from Octopus

The correlation of esterase and of acetylcholine content with responses to acetylcholine is confusing and raises a question of the significance of esterase and acetylcholine, which occur in many groups Good evidence that acetylcholine plays a part in neuromuscular transmission exists for annelids In the earthworm and leech the body wall is stimulated to contract by acetyl-

TABLE 3

*Cholinesterase in representative nervous and neuromuscular tissues*

| ANIMAL TISSUE | REFERENCE | Q Ch E mgm Ach HYDROLYZED BY 100 MGM. TISSUE PER HOUR AT MP |
|---|---|---|
| Vertebrates | | |
| Dog—brain cortex | (212) | 2–3 |
| Rat—brain cortex | (252) | 4 7* |
| Guinea pig—brain cortex | (252) | 4 6* |
| Guinea pig—brain cortex | (192) | 1 3 |
| Ox—brain cortex | (215a) | 1 5* |
| Ox—caudate nucleus | (215a) | 5 9* |
| Dog—lenticular nucleus | (212) | 70 0 |
| Dog—cer sympathetic ganglion | (212) | 14 0 |
| Cat—cer sympathetic ganglion | (116) | 36 0 |
| Cat—cer sympathetic ganglion | (272) | 28 4 |
| Guinea pig—cer sympathetic ganglion | (272) | 12 8 |
| Cat—sympathetic fibers | (272) | 3 7 |
| Dog—sympathetic fibers | (212) | 5 0 |
| Cat—sciatic nerve | (272) | 0 7 |
| Frog—sciatic nerve | (193) | 0 78–1 0 |
| Chick (9 day incubation) muscle | (213) | 3 0 |
| Chick (20 day incubation) muscle | (213) | 9 4 |
| Chick (21 day after hatching) muscle | (213) | 0 9 |
| Chick adult muscle | (213) | 0 4 |
| Frog—thigh muscle | (192) | 0 75 |
| Frog—sartorius (pelvic end) | (195 193) | 0 13 |
| Frog—sartorius (tibial end) | (195 193) | 0 4–0 8 |
| Lizard—muscle | (195) | 1 6–3 4 |
| Gymnotus—electric organ | (215) | 90–150 |
| Torpedo—electric organ | (215) | 150–300 |
| Raja—electric organ | (215) | 3–10 |
| Electrophorus—electric organ anterior end | (214) | 400–500 |
| Electrophorus—electric organ, posterior end | (214) | 100 |
| Arthropods | | |
| Homarus—abdominal chain | (215a) | 2 3* |
| Homarus—central ganglia | (212 194) | 15–25 |
| Limulus—central ganglia | (284) | 6 9* |
| Melanoplus—brain | (202) | 0 406 |
| Honeybee—brain | (257) | 16 5* |
| Astacus—nerve cord | (163) | ++ |
| Periplaneta—nerve cord | (203) | 0 12 |
| Melanoplus—nerve cord | (202) | 0 356 |
| Homarus—nerve fibers | (212) | 10 0 |
| Limulus—cardiac ganglion | (284) | 14 27 |
| Limulus—whole heart | (284) | 1 6 |
| Callinectes—whole heart | (284) | 1 63 |
| Pagurus—whole heart | (284) | 1 57 |
| Libinia—whole heart | (284) | 1 18 |
| Melanoplus—whole heart | (202) | 0 04 |
| Melanoplus—thoracic muscle | (202) | 0 134 |
| Melanoplus—femur muscle | (202) | 0 068 |
| Limulus—skeletal muscle | (284) | 0 144 |
| Homarus—skeletal muscle | (195) | 0 2–0 5 |

TABLE 3—*Continued*

| ANIMAL TISSUE | REFERENCE | Q Ch E mgm Ach HYDROLYZED BY 100 MGM TISSUE PER HOUR AT NTP |
|---|---|---|
| Arthopods—*Continued* | | |
| Homarus—skeletal muscle | (18) | 0 077 |
| Melanoplus—blood | (202) | 0 059 |
| Limulus—blood (serum) | (284) | 1 34* |
| Rat—blood | (252) | 1 37* |
| Guinea pig—blood | (252) | 3 51* |
| Molluscs | | |
| Modiolus—heart | (284) | 8 73 |
| Venus—heart | (284) | 0 176 |
| Eusepia—muscle | (18) | 0 076 avg |
| Eusepia—brain | (18) | 1 18 |
| Loligo—giant axon (whole) | (44) | 0 15 |
| Loligo—giant axon (sheath) | (44) | 0 42 |
| Loligo—unspecified ganglion | (215a) | 150 23* |
| Loligo—mantle nerve | (215a) | 0 70* |
| Annelids | | |
| Pontobdella—skin-muscle | (18) | 0 047 avg |
| Echinoderms | | |
| Asterias—radial nerve cord | (60) | 0 384 |
| Thione—radial bands | (60) | 0 820 |
| Holothuria—muscle | (18) | 0 0745 avg |
| Nemerteans | | |
| Cerebratulus—dorsal muscle | (281) | 6 86* |
| Flatworms | | |
| Prototyla—body (Dendrocoelum) | (60) | 9 3 |
| Planaria—body | (60) | ++++ |
| Ctenophores | | |
| Mnemiopsis—comb plates, etc | (60) | 0 |
| Coelenterates | | |
| Tubularia—heads | (60) | 0 267 |
| Tubularia—cleaned stalks | (60) | 0 312 |
| Metridium—whole | (60) | 0 034 |
| Metridium—oral disc | (60) | 0 009 |
| Metridium—muscle | (18) | 0 002 avg |
| Aurelia and Cyanea—epithelial sheet and related tissues | (60) | 2 positive out of 12 preparations |
| Sponges | | |
| Scypha—whole | (60) | 0 |
| Protozoa | | |
| Paramecium | (60) | 0 |

* QChE calculated from data given in reference

TABLE 4

*Acetylcholine content of selected tissues*

| ANIMAL TISSUE | REFERENCE | Ach CONTENT IN γ/gm. |
|---|---|---|
| Vertebrates | | |
| Dog—brain | (179) | 1 5-3 8 |
| Dog—brain | (66) | 0 4 |
| Cat—brain | (179) | 0 4-2 |
| Cat—basal ganglia | (83) | 0 4 |
| Cat—sympathetic ganglia (normal) | (52) | 10-20 |
| Cat—sympathetic ganglia (denervated) | (52) | 1-3 |
| Cat—sympathetic ganglia (normal) | (189) | 25 |
| Cat—sympathetic ganglia (denervated) | (189) | 7 |
| Horse—sympathetic nerve | (66) | 1 8-2 0 |
| Rabbit—outer intestinal layer (with myenteric plexus) | (318a) | 10-16 |
| Guinea pig—outer intestinal layer (with myenteric plexus) | (318a) | 16-20 |
| Arthropods | | |
| Crayfish—nervous system | (163) | 20 |
| Crayfish—ventral nerve cord | (279) | 9 8 |
| Crayfish—nerves | (285) | 3-12 |
| Crayfish—ganglia | (285) | 14-46 |
| Crayfish—blood | (285) | 0 7-1 1 |
| Homarus—nerve cord | (279) | 15 9 |
| Cancer—nerve cord | (279) | 3 1 |
| Callinectes—nerve cord | (279) | 6 6 |
| Carcinus—nerve cord | (163) | 2-4 |
| Palinurus—abdominal muscle | (9) | <0 1 |
| Cockroach—nerve cord | (203) | 70 |
| Insects (series of genera)—ganglia | (78) | 35-200 |
| Insects (series of genera)—head | (78) | 2 5-50 |
| Insects (series of genera)—blood | (78) | 0 |
| Molluscs | | |
| Helix Unio Anodonta—nerves and ganglia | (5) | ++ |
| Aplysia—esoph ganglion | (10) | 2-3 |
| Aplysia—foot | (10) | 0 3 |
| Aplysia—stomach | (10) | 0 6 |
| Pectunculus—body | (10) | 0 65 |
| Octopus—cerebral ganglion | (10) | 77 |
| Octopus—stomach and esophagus | (10) | 1 4 |
| Octopus—ovary | (10) | 0 2 |
| Octopus—testis | (10) | 0 |
| Octopus—mantle muscle | (10) | 0 7 |
| Octopus—blood | (10) | 0 |
| Annelids | | |
| Spirographis—body | (10) | 0 5-0 7 |
| Halla—body | (10) | 0 8 |
| Sipunculus—body | (10) | 0 8-0 9 |
| Sipunculus—muscle | (10) | 0 7 |

TABLE 4—*Continued*

| ANIMAL TISSUE | REFERENCE | Ach CONTENT IN γ/gm. |
|---|---|---|
| Ascidians | | |
| Ciona—body | (10) | 0 |
| Phallusia—body | (10) | 0 |
| Echinoderms | | |
| Holothuria—longitudinal muscle | (10) | 1 5-1 7 |
| Holothuria—intestine | (10) | 0 4 |
| *Coelenterates* | | |
| Rhizostoma—body | (10) | 0 |
| Alcyonum—body | (10) | 0 |
| Porifera | | |
| Leuconia—body | (10) | 0 |
| Siphonocula—body | (10) | 0 |

choline and sensitivity is increased by eserine The action of acetylcholine upon the earthworm body wall is abolished by nicotine (333) Summation of contractile response of the earthworm body wall to repetitive electrical stimulation is increased by eserine (47) Bacq and Coppée (16) found that eserine increased the muscular responses of Sipunculus, Hirudo, Arenicola, Aphrodite and Lumbricus to stimulation of the nerve cord They found no such effect when the eserine was applied to the nerve cord alone but they did get evidence that the leech body wall liberated acetylcholine when the nerve cord was stimulated. The crop and gizzard of the earthworm show spontaneous rhythmic contractions and acetylcholine at $10^{-9}$ superimposes a slow contraction on this rhythm (332) When the preparations are from earthworms which have been cooled the spontaneous contractions are absent but the response to acetylcholine persists, the ACh effect on the gut is abolished by atropine A spontaneously active preparation liberates into bathing fluid a substance which may be acetylcholine (4) From the preceding evidence and other contributing details it is likely that acetylcholine has something to do with neuromuscular excitation in somatic and visceral muscles of annelids

Molluscs appear to differ much in their responses For example, the slow adductor muscle of Pecten is not stimulated by acetylcholine (26), whereas the retractor muscle of the buccal mass in Helix does respond to the drug (253) In the cephalopods Octopus and Eledone acetylcholine and eserine have no effect on the stellate ganglion, the neuromuscular response of the mantle is not increased by eserine but the mantle muscle is sensitive to acetylcholine and removal of the stellate ganglion greatly increases the mantle sensitivity (12, 15) There was no increase by eserine of responses of foot and siphon muscles of Buccinium and Mya (16) Bacq (12) perfused the mantle of Eledone with eserinized saline, the perfusate contained acetylcholine but stimulation of the mantle nerve did not increase its Ach content

In the holothurian Stichopus the longitudinal muscles are sensitive to acetyl choline, responding to dilutions of $10^{-7}$ without eserine and to $10^{-9}$ after eserine (13) The mantle muscle of the ascidian Cynthia is stimulated by acetylcholine but the response is not potentiated by eserine (15) Bullock (personal communi cation) found that spontaneous contractions of the proboscis of the enteropneust, Saccoglossus, were greatly increased by acetylcholine That this sensitivity is not a property of all nerve nets was shown by the fact that the contractions of medusae were not affected by acetylcholine or eserine. Muscles of the actinians Calliactis and Metridium were also insensitive to acetylcholine $10^{-4}$, even after eserinization (12) In general, in the above groups of animals there is a rough correlation between response to acetylcholine and the existence of cholinesterase

Among the arthropods, on the other hand, except for heart and gut there is no clear evidence for any function of acetylcholine DuBuy (87) stated that the muscles of the crayfish claw are insensitive to acetylcholine, this has been con firmed by Bacq (11) and by Ellis, Thienes and Wiersma (92) The last named authors tested a variety of drugs and ions upon crustacean neuromuscular preparations and could find no evidence for humoral transmission Katz (166) and Waterman (315) had found an inverse relation between the response of crustacean muscle and magnesium content of the bathing saline but Ellis et al (92) did not confirm this There seems to be agreement among all these writers that curare, eserine, and acetylcholine have no effect upon neuromuscular re sponses of crustaceans

When applied to arthropod central nervous systems acetylcholine is also ineffective either with or without eserine except at concentrations as high as $10^{-3}$ in the crayfish (245) and in the cockroach (263) Bacq (11) injected large amounts (up to 40 mgm ) of acetylcholine into a crab (Carcinus and Palinurus) and observed no changes in reflex activity Welsh and Haskin (318) found a facilitation of autotomy in crabs injected with eserine or acetylcholine

Synaptic transmission in the last abdominal ganglion of the crayfish was studied by Prosser (243, 245) He stimulated sensory hairs on the uropods and telson mechanically and recorded simultaneously the impulses on both sides of the ganglion Flexion of one hair sent one impulse into the ganglion Stimula tion of several adjacent hairs, usually 4, was necessary to elicit one efferent impulse, i.e , spatial summation was required Ganglionic delay for the fastest units was 2–5 msecs The *sensory endings* followed repetitive stimulation up to 100 per second while the synapse failed to transmit faster than 10 per second Paired stimuli showed a non-functional recovery period in the ganglion of 0 1 sec. No after-discharge in the efferent response was ever observed A very different arrangement exists in the last abdominal ganglion of the cockroach where some sensory fibers pass directly through the ganglion while others synapse with giant neurones (250) The synaptic delay of the latter is 1–2 msec When the sensory fibers were stimulated repetitively a critical frequency was reached (40–50/sec at maximal intensities and 15/sec at submaximal intensities) where the postganglionic response diminished and its latency increased. Recovery of this response could then be brought about by suddenly increasing either the frequency or the intensity, or by interpolating a single extra stimulus on the

afferent side Temporal summation occurred only when the synapse was in an adapted state of diminished transmission Apparently a single sensory impulse was effective, hence no spatial summation was needed, but a given giant neurone could be excited by one of several converging sensory fibers

Prosser tested the effects of various drugs and salts upon ganglionic transmission in the crayfish preparation (245) Acetylcholine, physostigmine (eserine) and prostigmine were without effect upon summation or latency of the efferent response Treatment of the ganglion with a saline low in potassium did increase facilitation while high potassium decreased it reversibly The effect of potassium upon the asynchronous "spontaneous" activity of the nerve cord differed in intact and isolated cords (245, 247a, 264) When the cord was in situ, or within a half hour after removal from the animal, low potassium increased "spontaneous" activity while high potassium initially increased and then depressed it After a half hour of isolation in saline the potassium effect was reversed so that low potassium decreased "spontaneous" activity Neither stimulating nor depressing action of low potassium was antagonized by low calcium and only the depressing effect of high potassium was antagonized by high calcium Roeder (265) confirmed these findings and added the important fact that if acetylcholine is added to the saline in which the isolated nerve cord is kept the original response of the intact preparation to potassium persists The lobster nerve cord loses much of its Ach to a bathing medium (279) Thus it may be that the acetylcholine which exists in the nerve cord (285) is not a synaptic transmitter but that it sensitizes the ganglion cells to any depolarizing agents (such as K), and that acetylcholine may be lost rapidly from an isolated cord

The preceding evidence indicates that cholinergic systems are widespread, certainly in most groups above coelenterates The functions of acetylcholine in annelids may be more similar to its functions in vertebrates than in molluscs and arthropods The meaning of the high cholinesterase and acetylcholine concentrations in arthropod and cephalopod nervous systems is not clear

Various invertebrates produce other pharmacologically active agents besides acetylcholine, but their effects have not been much studied In the leech ventral nerve cord there are chromaffine cells which may produce adrenalin (112) Similar cells occur in the Limulus heart (R Snider, personal communication) Adrenalin stimulates the visceral musculature of some invertebrates (Holothuria (31), earthworm (44)) A possible synergistic action between low concentrations of adrenalin and acetylcholine has not been investigated, nor has the occurrence of adrenalin among lower invertebrates We mentioned previously the cells of the stellate ganglion of Sepia, which seem to have become endocrine (335) The Scharrers (273) have described neurosecretory cells in the brains of Nemerteans, Annelids, Molluscs, Arthropods and vertebrates

## CONCLUSIONS

The comparative physiology of nervous systems gives less information for tracing phylogenetic relations than does the comparative physiology of many

other systems. This is because (1) the basic nature of nervous conduction is a cellular problem, and (2) most of the general nervous properties evolved very early In the coelenterate nerve net for example there is already a tendency for increasing speed by through fiber tracts, there is facilitation in such high degree that it determines a sort of polarity Inhibition in the sense of an inhibitory state and central antagonism with reciprocal innervation of muscles are present in annelids and, assuming phylogenetic bifurcation below annelids to the two main lines of descent, inhibition may well be found among flatworms

The use of a thick myelin sheath and nodes is a vertebrate development, syncytial giant fibers probably arose twice—those with contributing cells scattered along the nervous system in the annelid-crustacean line, and those with contributing cells grouped together in the cephalopod molluscs Unicellular giant fibers are large neurones and grade into ordinary ones, such enlargement of neurones has occurred many times.

It is not clear why peripheral conducting systems or subepidermal networks have been replaced so completely by local reflex centers It is quite possible that the subepidermal plexus in the earthworm, in molluscan feet (but not in echinoderms) is even now vestigial

One is impressed with the closely interconnected evolution of central nervous and neuromuscular mechanisms In the coelenterate nerve net system there is little distinction as to whether facilitation is neuro-neural or neuro-muscular Some of the so-called peripheral conduction in worms may be muscular In the arthropods the neuromuscular junction with multiple innervation, facilitation, inhibition becomes more important than the central nervous system in grading motion

As one passes toward the most successful or "higher" invertebrates one sees increasing headwardness in the nervous system The sensory basis for this is clear, the integrative basis less so Learning in the sense of a reversal of a stereotyped response is probably a property of all animals whether they have a nervous system or not, as Jennings has pointed out Many of the alterations in feeding reactions obtained in coelenterates are undoubtedly sensory adaptation phenomena. In the earthworm conditioning was carried out by the chain of ventral ganglia Only in the verticalis complex of cephalopods, in the corpora pedunculata of certain insects (and possibly in those of lower arthropods and polychaete worms) is there anything comparable to the association areas of a vertebrate brain Technical difficulties of small size and fragility have prevented the study that these structures suggest

Not enough work has been done with pharmacologically active agents in invertebrates to draw conclusions regarding chemical mediators It is likely that there are cholinergic nerves beginning with flatworms and possibly with coelenterates.

Invertebrate nerves offer many useful preparations for the cellular physiologist A few examples are giant fibers for studying membrane phenomena, nerve fibers which remain depolarized for minutes (Crustacea), neuromuscular junctions in which facilitation persists for many seconds (coelenterates, annelids),

inhibitory nerve fibers that can be isolated from excitatory fibers, and muscle fibers which receive from two to five nerve axons each of which elicits a different response

I am indebted to Dr Theodore H Bullock for a critical reading of the manuscript

## REFERENCES

(1) Adrian, E D J Physiol 72 132, 1931
(2) Adrian, E D J Physiol 91 66, 1937
(3) Alverdes, F Ztschr f vergl Physiol 6 558, 1926
(4) Ambache, N , A S Dixon and E A Wright J Exper Biol 21 46, 1945
(5) Artemov, N M and T J Beckbulatov Bull Biol Med Exper U R S S 5 375, 1938
(6) Arvanitaki, A Arch Internat Physiol 49 209, 1939
(7) Arvanitaki, A , A Fessard and V Kruta Compt Rend Soc de Biol Paris 122 1204, 1936
(8) Ashworth, J H Phil Trans Roy Soc London B 200 427, 1909
(9) Bacq, Z M Arch Internat de Physiol 40 357, 1935a
(10) Bacq, Z M Ibid 42 24, 1935b
(11) Bacq, Z M Ibid 42 47, 1935c
(12) Bacq, Z M Ibid 44 174, 1936
(13) Bacq, Z M Ibid 49 20, 1939a
(14) Bacq, Z M Ibid 49 16, 1939b
(15) Bacq, Z M Ibid 49 25, 1939c
(16) Bacq, Z M and G Coppee Ibid 45 310, 1937
(17) Bacq, Z M and F P Mazza Ibid 42 43, 1935
(18) Bacq, Z M and D Nachmansohn J Physiol 89 368, 1937
(19) Baldi, E J Exper Zool 36 211, 1922
(20) Baldi, E Boll Istit Zool Univ Roma 2 1, 1924
(21) Baldi, E Boll di Zool Torino 9 65, 1938
(22) Baldus, K Ztschr wiss Zool 121 557, 1924
(23) Bardeen, C R Am J Physiol 5 175, 1901
(24) Barnes, T C Am J Physiol 107 447, 1934
(25) Barnes, T C , J P Sell and W Spofford Ztschr f vergl Physiol. 18 280, 1932
(26) Bayliss, L E , E Boyland and A D Ritchie Proc Roy Soc London B 106 363, 1930
(27) Bayliss, L E , S L Cowan and D Scott J Physiol 83 439, 1935
(28) Bear, R S and F O Schmitt J Cell and Comp Physiol 9 275, 1937
(29) Bear, R S , F O Schmitt and J Z Young Proc Roy Soc London B 123 496, 1937
(30) Benson, A A , J T Hays and R N Lewis Proc Soc Exper Biol Med 49 289, 1942
(31) Benson, E B and B R Lutz Collecting Net 8 119, 1933
(32) Bergren, W R and C A G Wiersma J Gen Physiol 22 193, 1938
(33) Bert, P Mem de la Soc Sci de Bordeau Quoted from Ten Cate (301), 1867
(34) Bethe, A Arch f mikr Anat 50 589, 1897a
(35) Bethe, A Pflüger's Arch 68 449, 1897b
(36) Bethe, A Arch f mikr Anat 51 382, 1898
(37) Bethe A Allgemeine Anatomie und Physiologie des Nervensystems Leipzig, Thieme, 448 pp , 1903
(38) Bethe, A Pflüger's Arch 224 793, 1930
(39) Bethe, A Ztschr f vergl Physiol 24 613, 1937

(40) Biedermann, W Sitzungsb d K Akad Wiss Wien Math Naturw Klasse 93: Abt 3 56 1886.
(41) Biedermann W Pflüger s Arch 111: 251, 1906
(42) Bishop G H and P Heinbecker Am J Physiol 94: 170 1930
(43) Blaschko H., McK Cattell and J L Kahn J Physiol 73: 25 1931
(44) Bozll, E J and D Nachmansohn Science 92: 513, 1940
(45) Bogue, J Y and H Rosenberg J Physiol 83: 21P 1934
(46) Bogue, J Y and H Rosenberg Ibid 87: 158 1936
(47) Botsford, E F Biol Bull 80: 299, 1941
(48) Bovard, J F Univ Calif Pub 18: 103 1918
(49) Bozler, E Ztschr f Vergl Physiol 4 37, 1926
(50) Bozler, E. Ztschr f Zellforsch u mikr Anat 5 244 1927a.
(51) Bozler E Ztschr vergl Physiol 5 371, 1927b
(52) Brown, G N and W Feldberg J Physiol 86 290 1936
(53) von Buddenbrock, W Biol Zentralbl 41 41 1921
(54) Budington R. A Am J Physiol 7 155 1902
(55) Bullock T H Biol Bull 79: 91 1940
(56) Bullock, T H J Cell and Comp Physiol 22 251 1943
(57) Bullock, T H J Comp Neurol 80 355 1944
(58) Bullock, T H J Neurophysiol 8 55, 1945a
(59) Bullock, T H Yale J Biol and Med 17 657 1945b
(60) Bullock T H and D Nachmansohn J Cell and Comp Physiol 20 239 1942.
(61) Buttendijk, F J Arch Néerl Physiol Ser IIIc 18: 24 1933
(62) Carlson, A. J J Exper Zool 1 269 1904
(63) Carlson, A J Am J Physiol 15 136 1906
(64) Caselli, A Pflüger's Arch 74 158 1899
(65) Celesia P Genova (quoted from Ten Cate, 301)
(66) Chang C and J H Gaddum J Physiol 79: 255 1933
(67) Child, C M Am Nat 26 1051, 1892
(68) Child, C M J Comp Neurol 57: 199, 1933
(69) Chinn, P and F O Schmitt J Cell and Comp Physiol 9 289, 1937
(70) Clementi A Zool Jahrb (Abt Allg Zool ) 31: 277 1912
(71) Cobb P Proc Nat. Acad Sci 4 234 1918
(72) Cole L J Biol Bull 24: 362 1913
(73) Collier, H O J J Exper Biol 15: 339, 1938
(74) Collier, H O J Ibid 16 286 1939a.
(75) Collier, H O J Ibid 16 300 1939b
(76) Coonfield, B R. J Comp Neurol 56: 1932
(77) Copeland M J Comp Psychol 10: 339 1930
(78) Corteggiani, E and A Serfaty C R. Soc Biol Paris 131: 1124, 1939
(79) Cowan S L. J Physiol 77: 27P, 1933
(80) Curtis H J and K S Cole J Cell and Comp Physiol 19: 135 1944.
(81) Day E. C J Exper Zool 28 307, 1919
(82) Demoor, J Arch de Zool Ser 2 Vol 9 1891
(83) Diesbit B B J Physiol 80 409 1934
(84) Dotterweich H Zool Jahrb Abt Allg Zool 44 399 1928
(85) Drew G J Exper Zool 5 311 1907
(86) Drzewina, A Compt Rend Soc Biol Paris 65 320 1908
(87) DuBuy, H G Am J Physiol 114 224 1935
(88) DuBuy, H G and G Coffee Am J Physiol 116: 282 1936.
(89) Eccles J C R. Granit and J Z Young J Physiol 77: 23P, 1933.
(90) Eggers, F Ztschr f vergl Physiol 1 579 1924
(91) Eimer, T Verhandl d phys med Gesellsch Würzburg N F 6: 137 1874
(92) Ellis C H C H Thienes and C A G Wiersma Biol Bull 83: 334 1942

(93) ERLANGER, J AND E A BLAIR Am J Physiol 110 287, 1934
(94) ERLANGER, J AND H S GASSER Electrical signs of nervous activity Philadelphia, 221 pp , Univ of Pa Press, 1937
(95) EWING, H Z Kansas Univ Sci Bull 12 305, 1904
(96) FAIVRE, E Ann des sci nat zool ser 4, vol 8 245, 274, 1857
(97) FEDELE, M Atti Accad naz Linzei Rome ser V, 32 98, 184, 1923 Quoted from TEN CATE (301)
(98) FRAENKEL, G Ztschr f vergl Physiol 16 371, 1932a
(99) FRAENKEL, G Ibid 16 394, 1932b
(100) FRAENKEL, G Ibid 16 444, 1932c
(101) FRIEDLÄNDER, B Biol Zentrabl 8 363, 1888
(102) FRIEDLÄNDER, B Pflüger's Arch 58 168, 1894
(103) FRIEDRICH, H Zool Jahrb Abt Allg Zool 52 537, 1932
(104) FRIEDRICH, H Ztschr vergl Physiol 18 536, 1933
(105) VON FRISCH, K Zool Jahrb (Abt allg Zool ) 35 1, 1914
(106) FRÖHLICH, F W Ztschr f allg Physiol 7 393, 1908
(107) FRÖHLICH, F W Ztschr f allg Physiol 10 384, 1910a
(108) FRÖHLICH, F W Ibid 10 384, 1910b
(109) FÜRST, M Pflüger's Arch 46 367, 1890
(110) FURUSAWA, K J Physiol 67 325, 1929
(111) GALANT, S Biol Zentralbl 40 335, 1920
(112) GASKELL, J F J Gen Physiol 2 73, 1919
(113) GASSER, H S AND J ERLANGER Am J Physiol 80 522, 1927
(114) GASSER, H S AND H GRUNDFEST Am J Physiol 127 393, 1939
(115) GELEI, J VON Ztschr Zellforsch u mikr Anat 22 244, 1935
(116) GLICK, D J Gen Physiol 21 431, 1938
(117) GRAHAM, H T AND J L O'LEARY J Neurophysiol 4 224, 1941
(118) GRAY, J J Exper Biol 16 9, 1939
(119) GRAY, J AND H W LISSMANN J Exper Biol 15 506, 1938
(120) GRAY, J , H W LISSMANN AND R J PUMPHREY J Exper Biol 15 408, 1938
(121) HADZI, J Arb aus der Zool Inst Wien 17 225, 1937
(122) HALL, D M AND C F A PANTIN J Exper Biol 14 71, 1937
(123) HANSTRÖM, B Vergleichende Anatomie des Nervensystems der wirbellosen Tiere Berlin, Springer, 628 pp , 1928
(124) HARDY, W B J Physiol 67 166, 1929
(125) HART, P C Arch Néerl de Physiol 9 1924
(126) HARVEY, E N Carnegie Inst Wash Pub no 132, 27, 1911
(127) HATAI, S Jap J Zool 1 1, 1922
(128) HECHT, S J Exper Zool 25 229, 1918a
(129) HECHT, S Ibid 25 261, 1918b
(130) HECK, L Lotos, 67/68 168, 1919-1920
(131) HEINBECKER Am J Physiol 117 686, 1936
(132) HERTER, K Ztschr f vergl Physiol 14 609, 1931
(133) HERTER, K Ibid 17 209, 1932
(134) HESS, W N J Morph and Physiol 39 515, 1924
(135) HESS, W N J Comp Neurol 68 161, 1937
(136) HODGKIN, A L Proc Roy Soc B 126 87, 1938
(137) HODGKIN, A L J Physiol 94 560, 1939
(138) HOFMANN, F B Pflüger's Arch 132 43, 1910
(139) HOFFMANN, P Ztschr f Biol 63 411, 1914
(140) HOLMES, S J J Comp Neurol 16 200, 1906
(141) HOLMES, W Phil Trans Roy Soc London B 231 293, 1942
(142) HOLMES, W , R J PUMPHREY AND J Z YOUNG J Exper Biol 18 50, 1941
(143) VON HOLST, E Zool Jahrb (Allg Zool ) 51 547, 1932

(144) VON HOLST, E Ibid 53: 67, 1933
(145) VON HOLST, E. Ztschr vergl Physiol 21: 395, 1934
(146) VON HOLST E Biol Rev 10 234, 1935
(147) HOPKINS, A E J Exper Zool 46: 263 1926
(148) HOWELL, C D J Exper Zool 81 231 1939
(149) HURSH J B Am. J Physiol 127 131, 1939
(150) HYDE, I J Morphol and Physiol 9 431 1894
(151) JANSEN, R Zool Jahrb Abt allg Zool 50: 51 1931
(152) JASPER, H S Arch Int de Physiol 41 281 1935
(153) JAWLOWSKI H Acta Biol Exper 3: 289, 1929
(154) JENKINS O P AND A J CARLSON Am J Physiol 8: 251, 1903a.
(155) JENKINS, O P J Comp Neurol 13 259, 1903b
(156) JENNINGS, H S U of Calif Publ Zool 4 53, 1907
(157) JOHNSON, G E J Comp Neurol 36: 323 1924
(158) JOHNSON, G E J Comp Neurol 42: 19 1927
(159) JORDAN H Ztschr f Biol 41: 196, 1901
(160) JORDAN, H Pflüger's Arch 131: 317 1910
(161) JORDAN, H Ztschr f allg Physiol 17: 146 1916
(162) JORDAN, H Allgemeine vergleichende Physiologie der Tiere Gruyter, Berlin, 761 pp 1929
(163) JULLIEN A. AND D VINCENT Compt Rend Soc Biol Paris 129: 845, 1938.
(164) JUST B Ztschr f vergl Physiol 2: 155, 1925
(165) KAMADA T AND H KINOSITA Proc Imp Acad Tokyo 13: 220 374 1937
(166) KATZ B J Physiol 87: 199 1936
(167) KEIGHLEY, G AND C A G WIERSMA. J Cell and Comp Physiol 17: 305, 1941
(168) KEPNER, W A. AND A RICH J Exper Zool 26: 83, 1918
(169) KINOSHITA T Pflüger s Arch 134: 501 1910
(170) KINOSITA H Jap J Zool 9 209, 1941a.
(171) KINOSITA H Jap J Zool 9 221, 1941b
(172) KLEMENSIEWICS R Sitzber Akad Wiss Wien Math Nat Klasse III 78: 7, 1878
(174) KNOWLTON F P AND C J CAMPBELL Am J Physiol 91: 19 1929
(175) KNOWLTON, F P Biol Bull 82: 207, 1942
(176) KNOWLTON F P AND A R MOORE Am J Physiol 44: 490 1917
(177) KOPEC, S Zool Jahrb Abt allg Zool u Physiol 36: 453, 1919
(178) KRIJGSMAN, B J Ztschr f vergl Physiol 28: 286 1941
(179) KWIATOWSKI, H Arch exper Path und Pharmakol 177: 154, 1934.
(180) LANGELOH H P Zool Jahr Abt allg Zool 57: 235 1937
(181) LAPICQUE L AND C VEIL Compt Rend Soc Biol Paris 93: 1590 1925
(182) LEVIN A J Physiol 63: 113 1927
(183) LOEB J Pflüger s Arch 56: 247 1894
(184) LUCAS K J Physiol 35: 326 1907
(185) LUCAS, K Ibid 51 1 1917
(186) LULLIES, H Pflüger s Arch 233: 584 1934.
(187) LUND, E. E Univ Calif Publ Zool 39: 35 1933
(188) MACDOUGALL, M S Biol Bull 54 471 1928
(189) MACINTOSH F C Arch Internat de Physiol 47 321 1938
(190) MACLENNAN, R R Arch f Protistenk 86: 191 1935
(191) MANGOLD E Ztschr f allg Physiol 5: 135, 1905
(191a) MARMONT, G AND C A G WIERSMA J Physiol 93 173, 1938
(192) MARNAT A AND D NACHMANSOHN J Physiol 89: 359 1937a
(193) MARNAT A AND D NACHMANSOHN Compt Rend Soc Biol Paris 125: 41, 1937b
(194) MARNAT A AND D NACHMANSOHN Ibid 125: 1005, 1937c
(195) MARNAT, A. AND D NACHMANSOHN J Physiol 92: 37 1938.
(196) MARSHALL. Studies from Biol Lab Owens College 1: 313, 1886

(197) MATULA, J Pflüger's Arch 138 388, 1911
(198) MAXWELL, S S Pflüger's Arch 67 263, 1897
(199) MAYER, A G Carnegie Inst Wash Pub no 47, 1, 1906
(200) MAYER, A G Carnegie Inst Wash Pub no 102, 115, 1908
(201) MCCRACKEN, I J Comp Neurol 17 262, 1907
(202) MEANS, O W J Cell and Comp Physiol 20 319, 1942
(203) MIKALONIS, S J AND R H BROWN J Cell and Comp Physiol 18 401, 1941
(204) MILICER, W Acta Biol Exper Warsaw 9 174, 1935
(205) MONNIER, A M AND M DUBUISSON Arch Int Physiol 34 25, 1931
(206) MOORE, A R Am J Physiol 76 112, 1922
(207) MOORE, A R J Gen Physiol 5 327, 1923a
(208) MOORE, A R Ibid 6 73, 1923b
(209) MOORE, A R Sci Rep Tohoku Imp Univ ser 6, Biol 193, 1933
(210) MOORE, A R Physiol Zool 9 240, 1936
(211) MORI, S Dobuts Zasshi, Tokyo 47 553, 1937
(212) NACHMANSOHN, D C R Soc Biol Paris 127 894, 1938
(213) NACHMANSOHN, D J Physiol 95 29, 1939
(214) NACHMANSOHN, D , R T COX, C W COATES AND A L MACHADO Neurophysiol 5 499, 1942
(215) NACHMANSOHN, D AND B MEYERHOFF J Neurophys 4 348, 1941
(215a) NACHMANSOHN, D AND M A ROTHENBERG J Biol Chem 158 653, 1945
(216) OLMSTED, J M D J Exper Zool 36 57, 1922
(217) O'LEARY, J , P HEIMBECKER AND G H BISHOP Am J Physiol 109 274, 1934
(218) PANTIN, C F A J Physiol 67 xliv, 1929
(219) PANTIN, C F A J Exper Biol 11 11, 1934
(220) PANTIN, C F A Ibid 12 119, 1935a
(221) PANTIN, C F A Ibid 12 139, 1935b
(222) PANTIN, C F A Ibid 12 156, 1935c
(223) PANTIN, C F A Ibid 12 389, 1935d
(224) PANTIN, C F A Ibid 13 111, 1936a
(225) PANTIN, C F A Ibid 13 148, 1936b
(226) PANTIN, C F A Ibid 13 159, 1936c
(227) PARKER, G H J Exper Zool 22 87, 1917
(228) PARKER, G H J Gen Physiol 1 231, 1918
(229) PARKER, G H The elementary nervous system Lippincott, Philadelphia, 229 pp 1919
(230) PARKER, G H J Exper Zool 31 475, 1920
(231) PARKER, G H J Cell and Comp Physiol 1 53, 1932
(232) PARKER, G H AND H M PARSHLEY J Exper Zool 11 361, 1911
(233) PAVLOV, I Pflüger's Arch 37 6, 1885
(234) PETIT, L Compt Rend Acad Sci Paris, 107, 1888
(235) POMPILIAN, M Compt Rend Soc Biol Paris, Series 11 1 51, 1899
(236) PRINGLE, J W S J Exper Biol 16 220, 1939
(237) PRINGLE, J W S J Exper Biol 17 8, 1940
(238) PROSSER, C L J Comp Neurol 59 61, 1934a
(239) PROSSER, C L Quart Rev Biol 9 181, 1934b
(240) PROSSER, C L J Cell and Comp Physiol 4 185, 1934c
(241) PROSSER, C L J Exper Biol 12 95, 1933a
(242) PROSSER, C L J Comp Neurol 62 495, 1935b
(243) PROSSER, C L J Cell and Comp Physiol 7 95, 1935c
(244) PROSSER, C L Cold Spring Harbor Symposia 4 339, 1936
(245) PROSSER, C L J Cell and Comp Physiol 16 25, 1940
(246) PROSSER, C L Biol Bull 82 145, 1942
(247) PROSSER, C L J Cell and Comp Physiol 21 295, 1943a

(247a) Prosser, C L Ibid 22: 131, 1943b
(248) Prosser, C L and J Z Young Biol Bull 73: 237 1937
(249) Pumphrey, R. J J Exper Biol 15 500 1938
(250) Pumphrey, R J and A F Rawdon-Smith Proc Roy Soc London B 122: 106 1937
(251) Pumphrey, R J and J Z Young J Exper Biol 15 453 1938
(252) Quastel, J H M Tenenbaum and A H M Wheatley Biochem J 30: 1668, 1936
(252a) Raabe, S Ztschr f vergl Physiol 26 611 1939
(253) Ramsay, J O J Exper Biol 17: 96 1940
(254) Reitschel, P E Zool Ans 111 109 1935
(255) Richards A G J N Y Ent Soc 51 55, 1943
(256) Richards A G Ibid 52 286 1944
(257) Richards A G and L K Cutkomp J Cell and Comp Physiol 26: 57 1945
(258) Richards O W Biol Bull 56: 32 1929
(259) Rilliant P Comp Rend Soc Biol Paris 111 631 1932
(260) Robertson, T B J Physiol 31: 410 1904
(261) Roeder K D Biol Bull 69 203 1935
(262) Roeder, K. D J Exper Zool 76 353 1937
(263) Roeder K D J Cell and Comp Physiol 14: 1 1939
(264) Roeder, K D Ibid 21: 295, 1941
(265) Roeder, K D Federation Proceedings 3: 40 1944
(266) Romanes G J Phil Trans 166 269 1876
(267) Romanes G J Ibid 167 659, 1877
(268) Ross D M and C F A Pantin J Exper Biol 17: 61, 1940
(269) Rushton, W A H Proc Roy Soc. London B 132 423 1945
(270) Sanders F K and J Z Young J Neurophysiol 3: 501 1940
(271) Sasse E. Ztschr f allg Physiol 13: 69 1912
(272) Sawyer, C W and W H Hollinshead J Neurophysiol 8 137, 1945
(273) Scharrer, E and B Scharrer Physiol Rev 25 171, 1945
(274) Schmitt, F O and R S Bear J Cell and Comp Physiol 9: 261 1937
(275) Schmitt, F O and R. S Bear. Biol Rev 14 27 1939
(276) Schmitt F O and O H Schmitt J Physiol 98: 26 1940
(277) Segaar, J Ztschr f vergl Physiol 10: 120 1929
(278) Serkoff P N Pflüger s Arch 237 631 1936
(279) Shallek W J Cell and Comp Physiol 26: 15 1945
(280) Smallwood W M and M T Holmes J Comp Neurol 43: 327, 1927
(281) Smith, C C., B Jackson and C L Prosser Biol Bull 79 377 1940
(282) Smith J E Roy Soc London Philos Trans B 227: 111 1937
(283) Smith, J E Biol Rev 20: 29 1945
(284) Smith C and D Glick. Biol Bull 77: 321 1939
(285) Smith R I J Cell and Comp Physiol 13 325 1939
(286) Sommer J Ztschr f Biol 100: 153 1940
(287) Steiner J Die Funktionen des Zentralnervensystems und ihre Phylogenese III Abt Die wirbellosen Tiere Braunschweig 1898
(288) Stough H B J Comp Neurol 40: 409 1926
(289) Stough H B J Comp Neurol 50: 217 1930
(290) Straub W Pflüger s Arch 79: 379 1900
(291) Taylor C V Univ Calif Publ Zool 19: 403 1920
(292) Taylor, C V *In* Protozoa in Biological Research G Calkins and F M Summers, editors p 191-270 Fibrillar systems in ciliates 1941
(293) Taylor, G W J Cell and Comp Physiol 15: 363 1940
(294) Taylor, G W Ibid 18 233, 1941
(295) Taylor, G W Ibid 20: 359 1942

(296) TEN CATE, J Arch Néerl de Physiol 12 327, 1928 a
(297) TEN CATE, J Ibid 12 529, 1928b
(298) TEN CATE, J Ibid 13 391, 1928c
(299) TEN CATE, J Ibid 14 1, 1929
(300) TEN CATE, J Ibid 15 242, 1930
(301) TEN CATE, J Ergebn der Physiol 33 137, 1931
(302) TEN CATE, J Arch Néerl de Physiol 21 562, 1936
(303) TEN CATE, J Ibid 23 136, 1938a
(304) TEN CATE, J Ibid 23 541, 1938b
(305) TONNER, F Zool Anz 133 125, 1933
(306) VAN HARREVELD, A AND C A G WIERSMA J Physiol 88 78, 1936
(307) VAN HARREVELD, A AND C A G WIERSMA J Exper Biol 14 448, 1937
(308) VAN HARREVELD, A AND C A G WIERSMA Ibid 16 121, 1939
(309) VON UEXKÜLL, J Ztschr Biol 31 1895
(310) VON UEXKÜLL, J Ergebn d Physiol 3 1, 1904
(311) VON UEXKÜLL, J AND L G TIRALA Ztschr f Biol 65 25, 1914
(312) VULPIAN, A Leçons sur la physiologie générale comparée du système nerveux Paris 1866
(313) WALLENGREN, H Lund's Univ Arskriff N F 9 1, 1913 Quoted from TEN CATE.
(314) WARD, J J Physiol 2 214, 1880
(315) WATERMAN, T H J Cell and Comp Physiol 18 109, 1941
(316) WEEL, P B VON AND S THORE Ztschr f vergl Physiol 23 26, 1936
(317) WELLS, G P J Exper Biol 14 117, 1937
(318) WELSH, J H AND H H HASKIN Biol Bull 76 405, 1939
(318a) WELSH, J H AND J E HYDE Proc Soc Exper Biol and Med 55 256, 1944
(319) WIERSMA, C A G Proc Soc Exper Biol and Med 38 661, 1938
(320) WIERSMA, C A G Arch Néerl Physiol 24 242, 1939
(321) WIERSMA, C A G Biol Symp 5 259, 1941a
(322) WIERSMA, C A G J Comp Neurol 74 63, 1941b
(323) WIERSMA, C A G AND C H ELLIS J Exper Biol 18 223, 1942
(324) WIERSMA, C A G AND R G HELFER Physiol Zool 14 296, 1941
(325) WIERSMA, C A G AND A VAN HARREVELD Arch Néerl de Physiol 20 89, 1935
(326) WIERSMA, C A G AND A VAN HARREVELD J Exper Biol 15 18, 1938a
(327) WIERSMA, C A G AND A VAN HARREVELD Physiol Zool 11 75, 1938b
(328) WIERSMA, C A G AND A VAN HARREVELD Ibid 12 43, 1939
(329) WIERSMA, C A G AND G MARMONT Physiol Zool 11 312, 1938
(330) WOOLLARD, H J AND J A HAARFMAN J Anat 73 559, 1939
(331) WOORTMANN, K Ztschr f vergl Physiol 4 37, 1926
(332) WU, K S J Exper Biol 16 184, 1939a
(333) WU, K S Ibid 16 251, 1939b
(334) YERKES, R M J Animal Behavior 2 332, 1912
(335) YOUNG, J Z Quart J Micr Sci 78 367, 1936
(336) YOUNG, J Z J Exper Biol 15 170, 1938
(337) ZHUKOV, E K Bull Biol Med Exper Moscow 3 297, 1937

# THE PHARMACOLOGY OF THE VERATRUM ALKALOIDS

OTTO KRAYER AND GEORGE H ACHESON

*The Department of Pharmacology Harvard Medical School, Boston, Massachusetts*

A review of the pharmacology of the veratrum alkaloids is justified at the pres ent time by several significant advances The chemical nature of these com pounds has been considerably clarified by recent work. The fact that they bear some resemblance to the cardiac glycosides has contributed to the interest in the action of the veratrum alkaloids on the heart The clinical use of crude preparations containing veratrum alkaloids has undergone a revival in obstetrical clinics, where eclampsia is again treated with these substances Parallel with this development there has been a revival of interest in the circulatory action of the veratrum alkaloids, and considerable clarification of certain circulatory phenom ena has been achieved. Finally, as a result of improved techniques and new data, the interpretation of the action of the veratrum alkaloids upon nerve and muscle has changed radically since this subject was last reviewed

Because of the emphasis on these advances, this review is of necessity different from previous reviews (Gasser, 83, Querido, 203, Boehm, 27, Biberfeld, 19) Hence some phases of the subject may better be pursued there than here The medical reader interested in the therapeutic aspect must be referred to the original clinical literature. Although occasional reference is made to important clinical observations, the clinical use of veratrum alkaloids will not be discussed, as it has not yielded fundamental contributions

One point seems of particular importance for future experimental work. To assure a steady advance in the understanding of the action of the veratrum alkaloids, it is indispensable that pure alkaloids be used instead of alkaloid mixtures whenever this is feasible. This alone will make possible a complete pharmacological analysis Individual veratrum alkaloids differ markedly in their biological action Hence the use of pure substances is imperative in physiological work in which veratrum alkaloids are employed as pharmacological tools Incidentally, only the study of the pure veratrum alkaloids will permit a correct evaluation of the practical usefulness of the individual alkaloids as well as of their mixtures, and thus make possible the development of a rational basis for their clinical use

I THE CHEMISTRY OF THE VERATRUM ALKALOIDS. The veratrum alkaloids are obtained from liliaceous plants belonging to the sub-order Melanthaceae The species most thoroughly investigated are *Veratrum album*, Linn , native to Europe, *Veratrum viride*, Aiton, native to the United States and Canada, and *Schoenocaulon officinale*, Gray, also called *Asagroea officinalis*, Lindley, or *Veratrum Sabadilla*, Retz , the Mexican or West Indian Sabadilla [1]

[1] The name hellebore is wrongly applied to the Veratrum species or to drugs obtained from these plants as the Helleborus species belong to the family Ranunculaceae The incorrect use of the name hellebore therefore should be discontinued in the scientific literature

Alkaloids are probably present in all parts of these plants The sources from which the chemically known substances have usually been obtained in the past are the roots and rhizomes of *Veratrum album* and *Veratrum viride*, and the seeds of *Schoenocaulon officinale*, the "Sabadilla seeds "

The chemistry of the veratrum alkaloids is now sufficiently well known to make possible the recognition of several well-defined pure substances which are probably built upon a common fundamental structure

*Veratrum album* and *Veratrum viride* were found by various authors (42, 128, 132, 133, 197, 198, 199, 200, 225, 226, 227, 234, 236, 262, 264) to yield some or all of the following crystalline alkaloids protoveratrine, germerine, jervine, rubijervine, pseudojervine, and veratrosine The seeds of *Schoenocaulon officinale* (24, 30, 76, 119, 172, 263) yield the crystalline cevadine, the amorphous veratridine, and small amounts of the crystalline cevine Table 1 contains the characteristics of the important alkaloids which are present in the plant, or, like protoverine, protoveratridine, germine, and veratramine, can be obtained as hydrolytic products

Some of these alkaloids are ester alkaloids which by hydrolysis under suitable conditions can be split into a basic part, or alkamine, and one or more organic acids

$$\text{Protoveratrine} + 3H_2O \rightarrow \text{Protoverine} + \begin{matrix}\text{acetic acid}\\ \text{methyl-ethyl-acetic acid}\\ \text{methyl-ethyl-glycolic acid}\end{matrix}$$

$$\text{Germerine} + 2H_2O \rightarrow \text{Germine} + \begin{matrix}\text{methyl-ethyl-acetic acid}\\ \text{methyl-ethyl-glycolic acid}\end{matrix}$$

$$\text{Veratridine} + H_2O \rightarrow \text{Cevine} + \text{veratric acid}$$

$$\text{Cevadine} + H_2O \rightarrow \text{Cevine} + \text{tiglic acid (or angelic acid)}$$

The ester alkaloid protoveratridine, according to Poethke (198), is a decomposition product of germerine, the hydrolysis of which may occur in two stages

$$\text{Germerine} + H_2O \rightarrow \text{Protoveratridine} + \text{methyl-ethyl-glycolic acid}$$

$$\text{Protoveratridine} + H_2O \rightarrow \text{Germine} + \text{methyl-ethyl-acetic acid}$$

Jervine, rubijervine, pseudojervine, veratramine, and veratrosine are non-ester alkaloids, or alkamines, like protoverine, germine, and cevine Pseudojervine and veratrosine differ from the other alkamines in that they are glycosidic alkaloids (132, 133) On hydrolysis, they split into d-glucose and the respective alkamines, jervine and veratramine, according to the following formulae

$$\underset{\text{Pseudojervine}}{C_{33}H_{49}O_8N} + H_2O \rightarrow \underset{\text{jervine}}{C_{27}H_{39}O_2N} + \underset{\text{d-glucose}}{C_6H_{12}O_6}$$

$$\underset{\text{Veratrosine}}{C_{33}H_{49}O_7N} + H_2O \rightarrow \underset{\text{veratramine}}{C_{27}H_{39}O_2N} + \underset{\text{d-glucose}}{C_6H_{12}O_6}$$

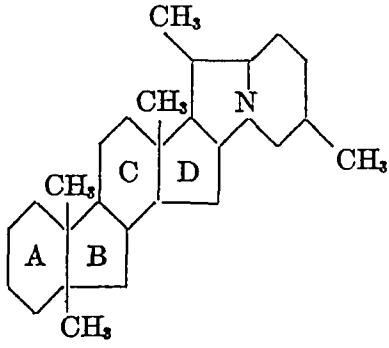
TABLE 1

*The empirical formulas, optical rotations and melting points of the known veratrum alkaloids*

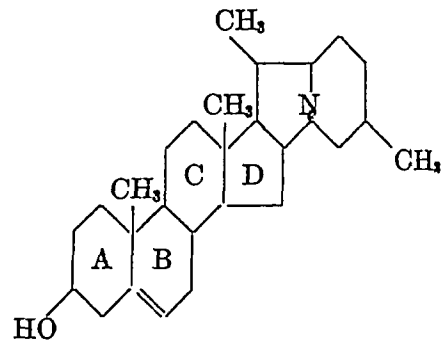
| NAME | EMPIRICAL FORMULA | ROTATION | MELTING POINT |
|---|---|---|---|
| Protoveratrine.. | $C_{32}H_{51}O_{12}N$ (42) | $[\alpha]_D^{25} = -8.5°$ (c = 1 99 in chloroform) (127) | Discolors above 270°, gradually decomposes at 273–276 (uncorr ) (42) |
| Germerine | $C_{27}H_{45}O_{11}N$ (45) | $[\alpha]_D^{25} = +10.8°$ (c = 1 99° in chloroform) (198) | 193–195 (corr ) melts with decomposition (197) |
| Veratridine | $C_{36}H_{51}O_{11}N$ (24) | $[\alpha]_D^{25} = +8.0$ (c = 4.0 in 96% ethanol) (24) | Softens when heated and melts over range of 160–180 (24) |
| Cevadine | $C_{32}H_{49}O_8N$ (172, 263) | $[\alpha]_D^{25} = +12 5°$ (c = 1 19 in ethanol) (172) | 199–201 (24) |
| Protoveratridine | $C_{32}H_{51}O_8N$ (45) | | 266–267 (corr ) Melts with decomposition (198) |
| Protoverine | $C_{27}H_{45}O_8N$ (131) | $[\alpha]_D^{25} = -12.0$ (c = 0 69 in pyridine) (131) | *No real melting point* Gradually softens to a resin between 195 and 200° (131) |
| Germine | $C_{27}H_{45}O_8N$ (45) | $[\alpha]_D^{25} = +5 0°$ (c = 1.03 in 95% ethanol) (45) | Gradually softens to resin between about 163–173 finally melts on heating to about 220 (45) |
| Cevine | $C_{27}H_{45}O_8N$ (24 76 172) | $[\alpha]_D = -17.52$ (c = 2.34 in ethanol) (172) | No sharp melting point sinters at 155–160 , becomes resinous at 165–170 , and is melted at 195–200 (76) |
| Rubijervine | $C_{27}H_{45}O_3N$ (129) | $[\alpha]_D^{25} = +19.0$ (c = 1.0 in ethanol) (129) | 240–242 (uncorr ) (129) |
| Jervine | $C_{27}H_{39}O_3N$ (130) | $[\alpha]_D^{25} = -147.0$ (c = 1.04 in ethanol) (130) | 237–238 (uncorr ) (130) |
| Veratramine | $C_{27}H_{39}O_2N$ (132 133) | $[\alpha]_D^{25} = -69.0$ (c = 0.98 in methanol) (133) | Melts at 204–207°C after preliminary sintering (133) |
| Pseudojervine | $C_{27}H_{41}O_3N$ (199) | $[\alpha]_D^{25} = -18.0$ (c = 0 4 in ethanol chloroform (199) | 304–305.5 (corr ) (199) |
| Veratrosine | $C_{33}H_{47}O_7N$ (132, 133) | $[\alpha]_D^{25} = -53.0$ (c = 0.255 in mixture of chloroform and 95% ethanol equal volumes) (132) | Discoloration and sintering especially above 230 softens above 235 , finally becomes a colored effervescent mass at 242–243 (132) |

[a] Germerine + 1 $H_2O$

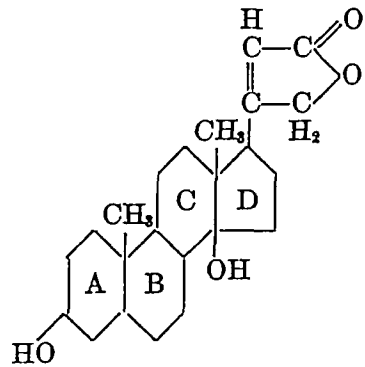
The chemical evidence suggests that the alkamines protoverine, germine, cevine, jervine, rubijervine and veratramine are $C_{27}$ compounds and are built upon a regular or modified sterol structure The analytical data support the assumption that the veratrum alkamines are closely related to the alkaloidal aglycones of the solanum species as well as to the aglycones of the cardiac glycosides (44, 46) The structural formulae illustrate the relation between the suggested ring system of cevine (I) (25, 42, 129, 223), the structure of solanidine (II) (46, 201, 213), and the structure of digitoxigenin (III) (79, 194)




I




II




III

The possibility persists that ring B of the alkamines other than rubijervine may be 5-membered The heterocyclic portion of the molecule in the tertiary bases has been established to be an octahydropyrrocoline derivative (251) The nature of the nitrogen containing portion of the molecule of jervine and of veratramine, which are secondary amines (133), remains to be determined All of the oxygen atoms are probably contained in hydroxyl or keto groups, the position of which is not yet ascertained (46)

That the chemical structure as formulated in (I) is correct and that the relation with solanidine holds has been confirmed by the conversion of sarsasapogenin to allosolanidanol-(3$\beta$) by Uhle and Jacobs (251)

For a valid interpretation of the biological work conducted in the past with

veratrum alkaloids it is important to recognize that the name veratrine[2] usually refers to a mixture of alkaloids obtained from Semen Sabadilla (30, 119) and consisting largely of veratridine, cevadine, and a small amount of cevine The relative proportion of the individual alkaloids apparently varies from batch to batch and the degree of variation is unknown The extracts of *Veratrum viride* or *Veratrum album* contain a large number of alkaloids, a few of which are chemically known, while the bulk of the alkaloidal material obtained from these plants remains to be separated and identified (see table 2)

As the habitat of the plant has a marked influence upon the composition of the alkaloidal mixture (197, 198, 199, 200, 225, 226, 234) in the various parts of the plant, it cannot be expected that extracts, obtained from specimens of the same species but grown in different locations should have the same pharmacological properties.

TABLE 2

*The alkaloids of Jugoslavian Veratrum album (Poethke, 197)*

| | ROOTS* | | RHIZOMES | | LEAF BASES* | |
|---|---|---|---|---|---|---|
| | Grams | % of total | Grams | % of total | Grams | % of total |
| Total alkaloids | 6 0 | 100 | 9 17 | 100 | 4 47 | 100 |
| Protoveratrine | 1 05† | 17 5 | 1 33† | 14 5 | 0 54 | 12 1 |
| Germerine | 0 45† | 7 5 | 1 26† | 13 7 | 0 8 | 17 9 |
| Jervine | 0 2 | 3 3 | 0 94 | 10 3 | 0 03 | 0 7 |
| Rubijervine | 0 2 | 3 3 | 0 04 | 0 4 | | |
| Pseudojervine | | | 0 6 | 6 5 | | |
| Amorphous alkaloids | 4 1 | 68 3 | 5 0 | 54 5 | 3 1 | 69 3 |

* One kilogram of dry powder was used to obtain the values

† These are approximate figures adapted from Poethke's data on pure protoveratrine and germerine and on mixtures of the two alkaloids

It is obvious, therefore, that neither the name veratrine (usually used for the total alkaloids from *Schoenocaulon officinale*), nor the name veratrum (usually used for extracts containing an unknown number of the total alkaloids from *Veratrum album* or *Veratrum viride*) refer to well-defined preparations of constant composition. But even in many cases in the past where it was implied by the author that pure substances were used for the experimental work, this must be considered doubtful unless specific data can be found indicating the identity of the veratrum alkaloids

II. TOXICITY QUANTITATIVE AND QUALITATIVE DIFFERENCES AMONG THE VERATRUM ALKALOIDS The naturally occurring ester alkaloids of the veratrum group are more powerful pharmacological agents than are the alkamines This calls to mind similar pharmacological facts for example, the difference between

[2] This is the only justifiable use of the name Veratrinum crystallisatum purissimum Merck is assumed to be cevadine

the solanaceous ester alkaloids, atropine and scopolamine, and their respective alkamines, tropine and scopine, in regard to their influence upon impulse transmission to autonomic effectors, or the difference in the pharmacological action of choline esters and choline While Falk (64) had already recognized that a considerable difference in toxicity exists between cevadine and cevine, quantitative toxicity experiments with pure alkaloids were made only recently (103, 154, 182) (see table 3) Protoveratrine so far has been shown to be the most toxic of the veratrum alkaloids The LD 50 in mice upon intravenous administration shows it to be on a molar basis 10 times more toxic than veratridine and 6000 times more potent than its own alkamine protoverine

TABLE 3

*The toxicity of the veratrum alkaloids (LD 50) in vertebrate animals*

| ANIMAL | WAY OF ADMINISTRATION | SUBSTANCE | MOL WEIGHT | LD 50 | | REFERENCE |
|---|---|---|---|---|---|---|
| | | | | mgm /kgm | Micromoles per kgm. | |
| Rana esculanta | Subcutaneous | Protoveratrine | 751 | 4 5 | 6 0 | (103) |
| Rana esculanta | Subcutaneous | Germerine | 679 | 9 0 | 13 2 | (103) |
| Rana temporaria | Subcutaneous | Protoveratrine | 751 | 13 5 | 17 9 | (103) |
| Rana temporaria | Subcutaneous | Germerine | 679 | 20 0 | 29 4 | (103) |
| Rat | Oral | Protoveratrine | 751 | 5 0 | 6 7 | (103) |
| Rat | Oral | Germerine | 679 | 30 0 | 44 1 | (103) |
| Rat | Subcutaneous | Protoveratrine | 751 | 0 6 | 0 8 | (103) |
| Rat | Subcutaneous | Germerine | 679 | 3 7 | 5 4 | (103) |
| Rat | Intraperitoneal | Veratridine | 673 | 3 5 | 5 2 | (182) |
| Rat | Intraperitoneal | Cevine | 509 | 67 0 | 131 0 | (182) |
| Mouse | Intravenous | Protoveratrine | 751 | 0 048 | 0 06 | (154) |
| Mouse | Intravenous | Veratridine | 673 | 0 42 | 0 63 | (151) |
| Mouse | Intravenous | Cevadine* | 591 | 1 0 | 1 7 | (153) |
| Mouse | Intravenous | Jervine | 425 | 9 3 | 21 9 | (154) |
| Mouse | Intravenous | Rubijervine | 413 | 70 0 | 170 0 | (154) |
| Mouse | Intravenous | Cevine | 509 | 87 0 | 170 0 | (154) |
| Mouse | Intravenous | Germine | 509 | 139 0 | 274 0 | (154) |
| Mouse | Intravenous | Protoverine | 525 | 194 0 | 367 0 | (154) |

* This was the alkaloid of Ikawa et al (113) and may not have been pure

The alkamines differ considerably in toxicity among themselves, the most toxic being jervine Chemically, jervine is a secondary amine while the others (with the exception of veratramine, which has not been studied) are tertiary amines Also, jervine is the strongest levorotatory compound of the alkamine group so far investigated Cevine is distinctly more effective than its isomer germine

Apart from and independent of the genuine toxicity of the alkamines, the toxicity of the ester alkaloids obviously is dependent upon the number of acyl groups in the molecule as well as upon the nature and probably also the position of the individual acyl groups This can be concluded from the limited and inaccurate toxicity studies on vertebrate animals reported in the literature (table 4)

It follows from the data of Heinz (109) (see table 4) that the introduction of an acetyl group and still more of a benzoyl group into the molecule of cevadine decreases notably toxicity in frogs as well as in rabbits Dibenzoyl cevine is still weaker in effect than benzoyl cevadine Haas' observations (103) on rats (tables 3 and 4) with subcutaneous injections of germerine, protoveratridine and germine indicate clearly that the removal of the methyl-ethyl glycolic acid from germerine reduces toxicity to $\frac{1}{333}$, and protoveratridine, the methyl-ethyl acetic acid ester

TABLE 4

*The toxicity of the veratrum alkaloids (approximate lethal doses) in vertebrate animals**

| ANIMAL | SUBSTANCE | LETHAL DOSE† | | REFERENCE |
|---|---|---|---|---|
| | | mgm./animal | mgm /kgm ‡ | |
| Rana | Protoveratrine | 0 2 | 6 0 | (256) |
| Rana | Cevadine | 0 05 | 1 5 | (109) |
| Rana | Cevadine (Sulfate?) | | 7 0 | (195) |
| Rana escul and temp | Cevadine | 0 5–1 0 | 15 0–30 0 | (166) |
| Rana | Acetyl Cevadine Hydrochloride | 1 0 | 30 0 | (109) |
| Rana | Benzoyl Cevadine | >10 0 | >300 0 | (109) |
| Rana | Dibenzoyl Cevine Acetate | 20 0 | 600 0 | (109) |
| Rana escul | Protoveratridine | | >750 0 | (103) |
| Rana escul | Germine | | 250 0–500 0 | (103) |
| Guinea pig | Cevadine | 0 5 | | (109) |
| Rabbit | Protoveratrine | | 0 11 | (256) |
| Rabbit | Cevadine | 1 0 | 0 5 | (109) |
| Rabbit | Cevadine | 2 7–4 2 | 1 3 | (166) |
| Rabbit | Acetyl Cevadine Hydrochloride | 50 0 | 25 0 | (109) |
| Rabbit | Dibenzoyl Cevine Acetate | >100 0§ | >50 0 | (109) |
| Rat | Protoveratridine | | >1000 0 | (103) |
| Rat | Germine | | 2000 0 | (103) |

* In all experiments the way of administration was by subcutaneous injection

† Unless otherwise mentioned the dosages refer to the base and not to the salt

‡ The boldface figures for lethal dose in mgm /kgm. were those actually reported The others were calculated from the reported values for the whole animal to get an approximate dose per kgm for the frog by multiplying by 30 assuming an average weight of 33 grams per frog for the rabbit by dividing by 2 assuming an average weight of 2 kgm per rabbit.

§ This dose caused only slight depression of the central nervous system

of germine, is but little more toxic than the alkamine germine itself Experiments on *Musca Domestica* L. (119) show veratridine to be a more potent insecticide than cevadine, while cevine dibenzoate and cevine have no insecticidal action.

The marked qualitative differences between the effects of the individual veratrum alkaloids are well illustrated by the observations on mice that died after the administration under the same conditions of an intravenous dose close to the LD 50 (164) (see table 3) The results with two ester alkaloids and five alkamines were as follows

*Protoveratrine* (relative LD 50 = 1) For a period of 90 seconds after the injection no signs of poisoning could be recognized A period of flaccid paralysis then followed suc ceeded in some cases by irregular respiration, in others by convulsions Death occurred within 2 to 4 minutes

*Veratridine* (LD 50 relative to protoveratrine = 10) Immediately after the injection respiration was decreased in rate and markedly disturbed in rhythm Death occurred with signs of respiratory failure within 1 to 4 minutes In some cases convulsions preceded death

*Jervine* (relative LD 50 = 350) After a latent period of 30 seconds, no appreciable sign of poisoning was noticed, marked motor excitation then started, leading to repeated convulsive seizures Death occurred in a convulsive attack within 10 minutes

*Rubijervine* (relative LD 50 = 3000) Immediately after the injection there was either mild motor excitation or a decrease in motor activity In some animals there was respira tory depression, in others, the outstanding symptoms were fits of tremor or convulsive seizures Death occurred within 30 seconds to 2 minutes in a convulsive attack or under the signs of respiratory failure

*Cevine* (relative LD 50 = 3000) Tremor and convulsive movements started immedi ately after the injection Within a few minutes the tremor became continuous, the animals lay prone, with legs and tail extended There was a very marked increase in motor excit ability to touch and especially to sharp, high-frequency noises Animals showing these signs died as a rule within 30 to 180 minutes A few animals had moderately severe convul sive seizures and a slow and labored respiration starting immediately after the injection These animals died within two minutes

*Germine* (relative LD 50 = 4500) Within a few seconds after the injection the animals began jumping about and had convulsive seizures Within 1 to 3 minutes they became unable to stand, lay on their sides and had continuous, severe convulsions Breathing was slow and irregular Death occurred within 1 to 3 minutes

*Protoverine* (relative LD 50 = 6000) After a latent period of about 30 seconds there were brief fits of coarse tremor, especially of the head In most animals, death occurred about 5 minutes after the injection following a brief period of convulsive movements Some animals had severe jerking movements, their respiration became slow and gasping, they died within 1 to 3 minutes

Other quantitative and qualitative differences among the alkaloids will be referred to in connection with the discussion of their effect upon the various physiological systems

III THE ABSORPTION, DISTRIBUTION AND ELIMINATION OF THE VERATRUM ALKALOIDS No adequate quantitative data are available on the absorption of the veratrum alkaloids from the mucosa of the intestinal tract or from other sites of application to the mammalian organism The experiments of Haas (103) on rats (see table 3) with oral and subcutaneous administration of protoveratrine and germerine indicate that approximately 90 per cent of the oral LD 50 becomes inactivated in some way during the passage from the intestinal tract to the sites of action responsible for the lethal effect, as the subcutaneous LD 50 of both alkaloids is only $\frac{1}{8}$ of the oral dose

The distribution of the alkaloids in the intact animal organism has not been studied In the isolated heart of a marine snail, *Aplysia limacina*, L which can take up relatively huge amounts of veratrine without being killed, Straub (243, 244) investigated the distribution between blood and heart tissue He exposed the heart to varying concentrations of veratrine in the blood and determined the final concentration in blood and heart by a comparison of the relative toxicity to

frogs of blood and of extracts of the heart tissue Straub concluded that a considerable accumulation of veratrine occurs readily in the heart muscle tissue. Equilibria are reached with low concentrations in the blood and high concentrations in the heart tissue. With increasing concentrations in the blood, the ratio of tissue concentration to blood concentration decreases Veratrine can be washed out of the muscle tissue of the Aplysia heart. According to Freundlich (77), Straub's data indicate that veratrine is adsorbed to the cell surfaces

Straub could not recognize any destruction of veratrine, although the alkaloid mixture was kept for 2 to 4 days in the tissue of the Aplysia heart

The fate of the veratrum alkaloids in the mammalian organism is unknown except for the scant evidence that a small fraction appears to be excreted by the kidney (202, 249), and by some of the excretory organs of the gastro-intestinal tract (118)

IV. THE ACTION OF THE VERTATRUM ALKALOIDS UPON THE RESPIRATORY SYSTEM. In the normal as well as in the anesthetized mammal all pure ester alkaloids of the veratrum series so far studied cause a decrease in the rate of respiration On the other hand, some of the veratrum alkamines even in large doses appear to be devoid of this property (154, 182)

The smallest active doses of the ester alkaloids, cevadine (109, 166), veratridine (182, 190), germerine (103) and protoveratrine (154, 256) bring about a transient slowing of respiration, larger doses lead to respiratory stoppage increasing in length proportional to the dose Qualitatively and quantitatively the respiratory effects of cevadine, germerine and veratridine appear to be similar In a large measure the respiratory arrest contributes to the lethal effect of fatal amounts

The minimal doses effective upon respiration are somewhat higher than the minimal doses influencing the circulatory system (section V, A and B) In the anesthetized dog (sodium pentobarbital) (190) no respiratory effect or occasionally a slight increase in respiratory rate is observed with the minimal doses of veratridine leading to a significant cardiodeceleration, or vasodilatation. With protoveratrine this difference is very pronounced Doses which in the dog under pentobarbital anesthesia cause marked cardiodeceleration and vasodilatation are without apparent respiratory action (154) The mechanism involved in the respiratory response to small doses appears to differ from that brought into action by large doses This is indicated by the experiments showing the rôle of the vagus nerves

Atropine sulfate in doses of 1 mgm per kilo in anesthetized dogs does not modify significantly the respiratory action of cardiodecelerator and depressor doses of veratridine. Vagotomy almost completely abolishes or prevents the respiratory action (190) After vagotomy larger doses, especially those designated in section V, D as pressor and cardioaccelerator doses, still cause respiratory arrest (190)

The experiments of von Bezold and Hirt (18) with veratrine and of Cramer (47) with veratrum viride make it obvious that part of the respiratory response is reflex in nature, while part appears to be due to central action The reflex action

can be demonstrated with small doses and in its full strength is difficult to obtain repeatedly, as the receptor mechanism appears to become readily irresponsive After this, doses larger than the minimal effective doses can be injected without effect, while further increase in dosage again leads to respiratory depression which seems to be independent of the functioning of the vagus nerves

Receptor areas for the inhibitory respiratory reflex are in areas served by the vagus nerves and probably are located in the viscera of the chest Exclusion of the depressor nerves in the rabbit does not abolish the response, while cutting the vagus nerves prevents the reflex respiratory action even when the depressor nerves are left intact (18) Similarly, cold block of the vagus in the cat (139) prevents the reflex respiratory effect as well as the vasodepressor effect of veratrine, while both promptly appear when conduction of the vagus nerves is restored by warming

No comprehensive and comparative study of the respiratory action of the pure veratrum alkaloids has been carried out so far The sites of action are not definitely established While it is unlikely that the doses leading to the reflex stoppage of respiration have a direct effect upon the striated muscles of the respiratory apparatus, this possibility exists with doses leading to death under the symptoms of respiratory failure Evidence is lacking, however, as to whether the intercostal muscles and the diaphragm, or their nerves are directly involved in the fatal respiratory disturbance

V THE ACTION OF THE VERATRUM ALKALOIDS UPON THE CIRCULATORY SYSTEM The studies on the circulatory action of the veratrum alkaloids in mammals have been concerned mainly with changes in blood pressure and heart rate in the intact animal and with effects upon the isolated heart On the basis of the available evidence a complete analysis of the very complex circulatory changes is not yet possible

It will facilitate the understanding of the mechanisms involved in the action upon blood pressure and heart rate if three at present clearly distinguishable effects are discussed in detail They are first, the depressor effect, second, the cardiodecelerator effect, and third, the pressor and cardioaccelerator effect The first and second as described in the following chapters can clearly be recognized if small doses of the veratrum alkaloids are used, they are not demonstrable unless the vagal mechanism is intact The third, on the other hand, can best be observed if the veratrum alkaloids are administered in large doses and if the vagus is partially blocked by giving atropine, or if the vagus nerves are completely interrupted, as for instance by cold block or by severing the nerves in the neck

A *The Depressor Effect of Small Doses of the Veratrum Alkaloids* 1 *Blood pressure decrease and vasodilatation* A transient decrease of blood pressure can be observed in mammals (man, dog, cat, rabbit) not only with veratrine, the alkaloid mixture from *Schoenocaulon officinale* (18, 138, 139), and with the alkaloid mixtures from *Veratrum viride* (47, 174, 196, 259, 261), *Veratrum album* (38, 39), and other *Veratrum* species (104), but also with the pure ester alkaloids, cevadine (166, 196), veratridine (182, 190), protoveratrine (154, 256), and germerine (103)

The active dosage range in dogs of 15 to 20 kgm body weight anesthetised with sodium pentobarbital is 0 02 to 0 1 mgm. veratridine (190) or 0 1 to 0 05 mgm. protoveratrine (154) injected intravenously close to the right atrium. Within this range the fall of blood pressure is proportional to the dose. An effect comparable to that of the highest dose of veratridine or protoveratrine can be obtained with the alkamine jervine on the basis of Wood's experiments in dogs (260) if doses of the order of 200 mgm. are injected intravenously None of the other alkamines has been tried in such large doses Cevine lacks the characteristic depressor property even in amounts up to 2000 times the minimal effective dose of its ester alkaloid, veratridine (182, 190)

The blood pressure decreasing action characteristic of the veratrum alkaloids is illustrated by the studies in dogs with the dosage range of veratridine (190) and protoveratrine (154) mentioned above Provided nothing interferes with the normal influence of the vagus nerves upon the circulatory system, there is, after the intravenous injection of veratridine close to the right atrium, a latent period of 4 to 12 seconds (average 7 3 sec in 8 expts ), mean carotid pressure then falls abruptly, reaches a minimum within 30 seconds, and within 2 to 3 minutes returns to the level of the control period or slightly less With protoveratrine the blood pressure fall is less abrupt, the minimum is reached within 1 to 3 minutes and the return to a level usually below the normal requires 10 to 30 minutes

This decrease in blood pressure, as a rule, is accompanied by a decrease in heart rate and, especially after veratridine, by a decrease in rate and amplitude of respiration (section IV) The cardiodecelerator as well as the respiratory response, if intense enough, contribute to the action upon the mean blood pressure However, artificial respiration (18, 154, 190, 196) which prevents the consequences of the respiratory effect, does not modify significantly the blood pressure response to veratrum alkaloids The experiments referred to below on the influence of atropine, or of severing of the vagi, upon the depressor effect, show that under conditions where there is little or no effect upon heart rate or respiration, depressor action can still be induced by the veratrum alkaloids Positive evidence of the independence of the depressor action from cardiodecelerator and from respiratory action is derived from the experiments of Krayer, Wood and Montes (155) on the innervated heart lung preparation of the dog In some of these experiments the head was perfused at a constant rate by means of a pump Only nervous connections existed between the head circulation and the artificially ventilated heart-lung preparation. Administration of veratridine into the heart-lung-circuit led to a decrease in blood pressure in the head circulation (see ref 155, fig. 4A)

These experiments demonstrate that a diminution of arterial resistance is involved in the blood pressure decrease caused by veratridine Similarly plethysmographic studies show an increase in the volume of various parts of the mammalian organism coincident with the fall of blood pressure caused by the intravenous injection of veratrine (139) or of veratrum viride (47, 259) Such increases in the capacity of the vascular bed have been observed in the liver (139), intestine (47) and kidney (47) of the cat, and in the hind leg and—although less con

sistently—in the spleen of the dog (259) This indirect evidence for vasodilatation has recently been substantiated by more direct observations The retinal arterioles of patients receiving veratrum viride dilate during the blood pressure fall (259) Direct quantitative measurements of blood flow in the femoral arteries of anesthetized dogs by means of a differential manometer show a marked absolute increase in blood flow during the blood pressure decrease caused by the intravenous injection of veratridine (190) or protoveratrine (154)

While vasodilatation has been demonstrated in various areas of the circulatory system of the mammal no direct quantitative measurements of alterations in blood flow have so far been made simultaneously in several areas It is not known what quantitative changes in the distribution of blood, in venous return, and in cardiac output result from graded doses of any one of the pure veratrum alkaloids

2 *The nature of the vasodilator response* During the blood pressure decrease the motor elements of the vessel wall, qualitatively at least, react like those of normal vessels to vasoconstrictor drugs (47) and to physiological constrictor stimuli (47, 139, 174) Thus injection of pituitary extract or epinephrine leads to a prompt rise in blood pressure, and asphyxiation leads to a reduction in the increased liver volume coincident with the blood pressure increase (139)

To decide the question whether the veratrum alkaloids have a direct effect upon the vessel wall or exert all their influence via the nervous system, Krayer and his collaborators (154, 190) carried out perfusion experiments in dogs with veratrine, veratridine, and protoveratrine One hind leg of a "recipient dog" was perfused from a "donor dog" by connecting one carotid artery of the "donor" with the femoral artery of the "recipient" and returning the blood from the femoral vein of the "recipient dog" to an external jugular vein of the "donor dog " All the remaining vascular connections between the perfused leg and the trunk of the "recipient dog" (with the exception of bone and nerve vessels) were interrupted by means of an ecraseur leaving intact the nerve connections via the main trunks of the mixed nerves Continuous blood flow measurements were recorded simultaneously from both femoral beds of the "recipient dog " The intravenous injection of the alkaloids into the "recipient dog" outside the perfused leg resulted in a characteristic increase in blood flow in the leg perfused from the "donor dog" as well as in the leg which was in normal vascular and nervous connection with the trunk of the "recipient dog " On the other hand, no response could be obtained by injections of the alkaloids into the arterial blood supply from the "donor dog" to the perfused leg of the "recipient dog " The dosage range of veratridine was 0 01 mgm to 0 5 mgm leading to concentrations of from 1 150,000 to 1 3000 during the intra-arterial injection With protoveratrine the doses ranged between 0 015 mgm and 0 075 mgm leading with the lowest dose to approximately 1 100,000, with the highest dose to 1 25,000 in the artery Thus doses leading to concentrations which might conceivably arrive at the entrance of the femoral vascular bed following the intravenous injection as well as doses many times higher proved ineffective upon direct intra-arterial administration

Perfusion experiments on isolated vascular beds, undertaken to study the direct

action of the veratrum alkaloids upon the vessels, have not yielded consistent results regarding the vasodilator effect. In the Trendelenburg preparation of the frog a slight increase in perfusion rate was observed (151) with concentrations below 1 100,000, higher concentrations reduced the flow In the hind legs of mice and rats or in the rabbit ear perfused with Ringer solution the minimal active concentrations of 1 200,000 or above caused only constriction of the vessels. Willson and Smith (259), however, observed increase in perfusion rate of the isolated kidney, ear, or hind leg of the rabbit perfused with Ringer solution when veratrone—a mixture of alkaloids of *Veratrum viride*—was injected The effect was more consistent when the rate of flow was reduced by the previous administration of epinephrine These experiments on isolated vascular beds need corroboration with pure veratrum alkaloids

The perfusion experiments in the innervated femoral bed of dogs justify the conclusion that the vasodilator action of small doses of veratrum alkaloids cannot be explained by a direct influence upon the vessel wall The action is transmitted to the motor effectors of the vessels predominantly, if not altogether by way of nervous pathways reaching the femoral vascular bed through the mixed nerves of the leg. The nature of the nerve fibers involved in the reaction has not been identified.

3 *The rôle of the central nervous system* The rôle of the central nervous system in the vasodepressor response to small doses of veratrum alkaloids is not entirely clarified There is no conclusive evidence of a direct inhibitory action upon the vasomotor center Doses of veratrine hydrochloride below 2 micrograms placed into the fourth ventricle of the cat are without any effect, while 2 to 5 micrograms cause increase in blood pressure and acceleration of heart rate The injection into the vertebral artery of the cat of doses of 10 to 20 micrograms of veratrine hydrochloride is either without effect or is followed by a slight rise in blood pressure and increase in heart rate. When injected into the jugular vein these doses have a distinct vasodepressor and cardiodecelerator action (209) These experiments support the assumption, first put forward by von Bezold and Hirt, that the vasodepressor response to small doses of veratrum alkaloids is not central but reflex. This is borne out by the studies on the afferent pathways and receptor areas involved in the vasodepressor response

4 *The rôle of the vagus* The blocking of cholinergic muscarinic effects by atropine in the cat (47, 139) and in the dog (174, 190) does not prevent the fall in blood pressure caused by veratrum viride (47, 174), veratrine (139) or veratridine (190) In the experiments of Moe, Bassett and Krayer (190) in dogs 1 mgm per kgm of atropine sulfate largely abolished bradycardia and had no effect upon the respiratory response, increase in femoral arterial flow still occurred and blood pressure fell although not quite as low as in control observations

Interruption of the conductivity of the vagus nerve by cutting or cooling has a more pronounced effect than atropinization Veratrum viride was found to be inactive in the cat after cutting the vagus nerves (47) Cold block of the vagus nerves prevented the blood pressure fall after veratrine in the cat (except after the first injections in some experiments) while the drop in pressure promptly occurred

when conduction was restored by warming the nerves (139) Conversely, after veratrine had established blood pressure decrease and increase in liver volume in the cat, cold block of the vagus immediately raised the blood pressure and caused a reduction of the liver volume Vagotomy below the heart did not prevent the vasodepressor response in the cat (139)

While interruption of the conductivity of the vagus in the neck markedly interferes with the vasodepressor effect, it does not abolish it In MacNider's experiments (174) with veratrum viride in the dog, the blood pressure did not fall to the same extent after vagotomy as before In seven out of ten experiments in dogs, Moe, Bassett and Krayer (190) found the depressor response to veratridine markedly diminished by vagotomy but not abolished altogether in any case This occurred in spite of the fact that in the majority of the experiments bradycardia and respiratory effects were either completely or almost completely prevented In three experiments of this series the relative vasodilator effect in the femoral arteries was as marked as before vagotomy Areas not served by the vagus must therefore contribute to the vasodepressor action

5 *Non vagal afferent pathways* If veratrine was administered into the circuit of the carotid sinus area of the dog, exteriorized according to Heymans (114), decrease of blood pressure and bradycardia occurred in the dog Occlusion with lycopodium of the vessels of the glomus caroticum prevented both effects (139) In the rabbit von Bezold and Hirt (18) could not abolish the depressor response to veratrine by cutting the depressor nerves, whereas the response was absent when the vagus nerves were cut and the depressor nerves left unimpaired While the carotid sinus nerves carry afferent fibers for the vasodepressor reflex, the depressor nerves presumably do not contribute to the reaction

6 *The receptor areas located in the heart and lungs* In order to demonstrate that a veratrum alkaloid confined to the heart and lungs can by a nervous mechanism cause the characteristic action in a distant vascular bed, Krayer, Wood and Montes (155) used the innervated heart-lung preparation of the dog The head was perfused with a pump at a constant rate No vascular but only nervous connections existed between the head-circuit and the heart-lung-circuit Placing veratridine into the heart-lung-circuit caused a decrease in blood pressure in the head-circuit

Jarisch and Richter (140) attempted to localize more precisely the receptor areas in the heart and lungs from which vasodepressor effects can be elicited They systematically interrupted various sections of the nerve supply of the heart and lungs of the cat and tested whether blood pressure decrease with veratrine could still be obtained They either cut the nerve branches or, in the immediate vicinity of the epicardium, destroyed the nerve tissue, or interrupted its conductivity by the application of local anesthetics or nerve poisons (procaine, phenol or veratrine) to special areas of the surface of the heart Afferent pathways for the depressor response were not found in the sympathetic nerves, or in the aortic nerves, but exclusively in the vagi While there were contributory afferent fibers coming from the lungs (208), the veratrine depressor response originated predominantly in the heart The pathways mainly run in those branches of the cardiac nerves not carrying efferent inhibitory fibers Afferent fibers coming

from the region of the ventricles were found to be of great importance for the circulatory reflex. These observations appear to resolve the disagreement between von Bezold and Hirt (18) who assumed that the impulses came from the heart only, and Cramer (47) who suggested that the lungs were their exclusive origin.

7 *Tachyphylaxis* Veratridine in the intact dog may be injected repeatedly with little or no diminution of depressor, or vasodilator responses, provided the doses are near the minimal effective dose, if larger doses are used, the effect of subsequent doses becomes less or may even be absent Protoveratrine, unlike veratridine, leads to marked weakening of the response even with the smallest effective doses (154, 190) According to Jarisch and Richter (140), the depressor reflex originating in the heart of the cat is less subject to tachyphylaxis than circulatory reflexes from other receptor areas.

8. *Summary* The evidence presented points to the following conclusions The mechanism mainly operative in the vasodepressor response of the mammalian organism to small doses of veratrum alkaloids is a vasodilatation of reflex nature. Most of the afferent pathways of the reflex run in the vagus nerves, none apparently in the aortic nerves and in the sympathetic outflow of the autonomic nervous system As, in the dog at least, vagal section in most cases reduces the magnitude of the response without abolishing it, areas not served by the vagus must contribute to the effect, the glomus caroticum appears to be one of these. According to Jarisch the most important receptor area for the reflex is in the heart, as von Bezold and Hirt suggested The evidence for the ventricular origin of an important section of afferent fibers needs corroboration While there are receptor areas also in the lungs, they are not the exclusive origin of the reflex, as Cramer assumed

B *The Cardiodecelerator Effect of Small Doses of the Veratrum Alkaloids* Small doses of the veratrum alkaloids, which upon intravenous injection in the mammal lead to the reflex vasodepressor action described in the previous chapter, as a rule cause a decrease in heart rate. Within the dosage range stated above for veratridine and protoveratrine in the dog the intensity of the bradycardia is proportional to the dose. There are considerable differences between the two alkaloids regarding the time-characteristics of their cardiodecelerator action When injection is made into or close to the entrance of the right atrium veratridine bradycardia (190) begins after a latent period of a few seconds, reaches its maximal intensity within 30 seconds, and lasts up to five minutes After protoveratrine the latent period lasts between 40 and 120 seconds, bradycardia develops gradually, the minimal heart rate is reached within one to three minutes, and the duration of action extends over ten to thirty minutes or more (154)

1 *Central versus reflex nature of the cardiodecelerator effect.* It has long been known that vagotomy abolishes or prevents the cardiodecelerator effect and that the administration of large doses of atropine greatly reduces the effect. While some of the investigators explain the lowering of the heart rate as due to central vagal stimulation (18, 115, 190), others consider it to be mainly the result of a reflex vagal stimulation (47, 139, 209)

The apparent discrepancy in regard to the central versus reflex nature of the

cardiodecelerator effect has been largely resolved by the experiments of Kr Wood and Montes (155) with veratrine and veratridine, and of Krayer, Moe Mendez (154) with protoveratrine The innervated heart-lung preparatic the dog was arranged in these experiments in such a way that the head circula was separated completely from the heart circulation and there were only ner connections between the two circuits Doses of five micrograms of veratridi protoveratrine injected into the heart-lung-circuit (total blood volume app mately 800 cc ) caused a decrease in heart rate of about 50 per cent, which did occur when the vagi were cut (In the ordinary heart-lung preparation dos this order are without effect upon heart rate ) Upon injection into the cepl circulation, doses of 10 to 70 micrograms of veratridine or 10 to 30 microgran protoveratrine also resulted in cardiac slowing, which, as was shown in ex ments with veratridine, could still be obtained after denervation of the car sinus, although the effect appeared to be less in intensity The alkamine ce given in doses of up to approximately 2 mgm into the heart-lung-circuit, or the head-circuit, did not affect the heart rate

Jarisch and Richter (139) report that marked bradycardia could be elicite the dog, at least with the first doses, when veratrine was placed into the isol carotid sinus perfused according to Heymans (114)

Provided there are no other receptor areas in the head region of the innerva heart-lung preparation after denervation of the carotid sinus, it must be assu on the basis of these experiments that the carodiodecelerator action of the v trum alkaloids in the mammal is caused in part by reflex and in part by cen vagal stimulation

It has been suggested (139) that a reflex inhibition of accelerator impulses r contribute to the cardiodecelerator action in the intact animal This wo explain the much greater difficulty of preventing the bradycardia of small do of the veratrum alkaloids by atropine as compared to complete interruptio afferent as well as efferent vagal fibers, but definite proof for this assumptio lacking

2 *Qualitative difference between veratridine and protoveratrine* In the in vated heart-lung preparation, as in the intact animal, a difference is apparen the time-characteristics of the cardiodecelerator response when the effect veratridine is compared with that of protoveratrine This is noticeable when alkaloids are administered into the heart-lung-circuit as well as when they injected into the head circulation The slowness of the development of max bradycardia is particularly striking when protoveratrine is injected into cephalic circuit

3 *Tachyphylaxis* The reflex decrease in heart rate originating in recep areas of the heart and lungs and of the carotid sinus cannot be obtained repeate with the veratrum alkaloids unless doses are used close to the minimal effect dose (47, 139, 154, 155, 190) Larger initial doses diminish or may altogeth prevent the response to a subsequent dose In case of veratridine it has be shown that this must be due mainly to a change in the receptor mechanism, cephalic administration in the innervated heart-lung preparation still leads

cardiodeceleration, when the injection into the heart-lung-circuit is no longer effective. The central action on the other hand does not appear to be weakened as readily

C *The Bezold Effect* As has been shown in section V, A and B, the reflex vasodilatation and cardiodeceleration caused by the veratrum alkaloids originates predominantly in the viscera of the chest According to Jarisch and Richter the most important part of the impulses for the vasodepressor reflex come from the myocardium of the ventricles and are carried exclusively by afferent fibers of the vagus nerves. For this reflex of cardiac origin they (138) propose the name Bezold effect, because it was A. von Bezold who explained by such a reflex mechanism the vasodepressor action of veratrine, which he discovered in 1867

Von Bezold and Hirt considered the possibility that the effect of the veratrum alkaloids might be due to a change in sensitivity of pressoreceptor endings to their adequate stimuli. The receptors might be specific chemoreceptors, however, and biochemical changes might lead to an increased discharge of the receptors It is of interest in this connection that the veratrum alkaloids are not the only substances capable of eliciting the Bezold effect According to Jarisch and his collaborators (137, 142), other substances, especially active principles of *Viscum album*, L., also reveal apparently the same circulatory reflex.

As far as the veratrum alkaloids are concerned, it has been pointed out above that the ester nature appears to be essential for the reaction, and that the mechanism by which the esteralkaloids act is subject to tachyphylaxis Following the concept of Bacq (11) (section VI, L) Jarisch (135) put forward the hypothesis that the receptors in the heart responsible for the Bezold effect are sensitized by the veratrum alkaloids to the influence of potassium ions released during the activity of the heart muscle Amann and Jarisch (8) present evidence that in cats the intravenous injection of otherwise ineffective doses of potassium ion causes circulatory effects similar to the Bezold effect after the animals have been given several doses of 0 05 to 0 1 mgm of veratrine hydrochloride The vasodepressor and vasodilator effect can be abolished or prevented by cold block of the vagus and can no longer be elicited after local anesthesia of the epicardium by procaine. Similar effects are obtained with barium ion and rubidium ion, as well as with substances like sodium citrate and sodium oxalate, which shift the potassium-calcium ratio in favor of the potassium ion Conversely injection of calcium salts has an antagonistic effect.

Extensive corroborative evidence is required to establish the concept of the Bezold effect, especially as Jarisch (135, 138) is inclined to ascribe to it a considerable importance in the control of the distribution of the blood in the mammalian circulatory system under physiological and pathological conditions It must be emphasized that it is a complicated task to separate experimentally chemo- and pressoreceptor areas in the myocardium of the ventricles from those already recognized in the large vessels or attached to them, and this task cannot yet be considered to have been satisfactorily accomplished

D *The Pressor and Cardioaccelerator Effect of the Veratrum Alkaloids* The

intravenous injection of more than 0 1 mgm of veratridine in dogs of 15 to 20 kgm causes a blood pressure fall to very low levels and the pressure may fail to recover (190) After doses of protoveratrine of the same order there is usually a secondary increase in blood pressure above the normal level (154, 256)

1 *The simulated vagus block caused by protoveratrine* As Watts Eden (256) already observed, protoveratrine leads to a temporary decrease or abolition of the normal response to electrical stimulation of the peripheral end of the severed vagus nerve Doses of 0 1 to 0 3 mgm of protoveratrine (144) injected into dogs of 15 to 20 kgm cause a complete abolition of the response, and recovery is still incomplete after three hours The electrocardiographic analysis reveals that during vagal stimulation the normal pacemaker is depressed and an ectopic rhythm below the A V node is established Rothberger and Winterberg (222) observed this phenomenon with several substances In contrast to the action of atropine this effect of protoveratrine is exerted on the automatic properties of the myocardium rather than on either the vagus nerves or the mechanism of humoral transmission Veratridine in doses of 0 1 to 0 2 mgm lacks this action The action of protoveratrine in causing a simulated vagal block may explain in part the weakening at least of the cardiodecelerator response when repeated doses are given

2 *The time-characteristics of the pressor and cardioaccelerator effect* After vagotomy doses of veratridine between 0 1 and 0 5 mgm in the dog still cause a blood pressure decrease, but this becomes less as the dose increases Doses between 0 5 and 1 0 mgm after vagotomy, as well as after blocking of the vagus with atropine, cause an increase in carotid pressure up to 50 mm Hg or more The pressure begins to rise within 15 to 30 seconds after the intravenous injection close to the right atrium, reaches the maximum within one to three and one half minutes, and gradually returns to normal within six to twelve minutes The heart rate always increases as the pressure rises, and rates can be reached as high as 255 per minute from an initial rate of 162 A similar hypertension and tachycardia results from doses of 0 1 to 1 0 mgm of protoveratrine after the vagus has been cut, or after initial doses of the alkaloid have caused a simulated vagal block Unlike the ester alkaloids the alkamine cevine even in doses of 40 mgm does not have any characteristic vasopressor or cardioaccelerator action

3 *The vasoconstrictor response* Coincident with the increase in blood pressure caused by the ester alkaloids, veratridine or protoveratrine (154, 190), the blood flow in the femoral arteries increases However, this is followed by a decrease of flow at the height of the blood pressure response, and throughout the blood pressure change the ratio of pressure to flow is increased, sometimes more than doubled, suggesting actual constriction Vasoconstriction occurs in the acutely denervated limb and denervated femoral artery as well as in the innervated limb At least two factors may therefore partake in this vasopressor response 1, vasoconstriction by local action of the veratrum alkaloids upon the vessel wall, or vasoconstriction by substances (like epinephrine) released by the veratrum alkaloids and carried to the femoral bed by the blood stream, 2, an action of the veratrum alkaloids upon the nervous system leading to central vasomotor stimulation

Vasoconstriction ascribed to local action of veratrine hydrochloride was observed by Kobert (148) in the isolated hindquarters of dogs perfused with dog's blood at 38°C, and in the isolated liver of the dog perfused from the hepatic artery Concentrations of veratrine hydrochloride 1 10,000 were administered for 3 minutes into the leg arteries, 1 20,000 for 1 and 2 minutes and 1 40,000 for 5 minutes were given into the hepatic artery, causing a marked decrease in outflow from the organs which lasted from 9–15 minutes after the period of exposure In the hind legs of mice and rats and in the isolated rabbit's ear perfused with Ringer solution, vasoconstriction with veratrine (151), although noticeable at lower concentration, was not constant below 1 20,000 In the hind legs of the frog (prepared according to P Trendelenburg) veratrine 1 20,000 (151), germerine 1 40,000 (103) and protoveratrine 1 100,000 (103) caused vasoconstriction (see also section VI, A2, p 411)

These observations on isolated organs cannot satisfactorily explain the hypertensive action of veratridine and protoveratrine in dogs described above, for the injection of as much as 0.5 mgm of veratridine into the arterial supply from a "donor dog" to the perfused leg of a "recipient dog" fails to cause any constriction On the other hand, the intravenous injection of 0 5 to 1 0 mgm veratridine into the vagotomized "donor dog" causes an increase in "donor" arterial pressure with an initial flow increase in the perfused limb of the "recipient dog," but as the pressure in the "donor dog" reaches its maximum, flow rapidly decreases in the perfused limb of the "recipient dog" This indicates that a vasoconstrictor substance other than veratridine must reach the perfused femoral bed of the "recipient dog" from the "donor dog"

Conversely, if doses of 1 mgm of veratridine or protoveratrine are injected into a vagotomized "recipient dog," blood flow in the femoral bed of a leg with nerves intact but perfused from a "donor dog" shows a long lasting increase (This undoubtedly is in part caused by the reflex vasodilator effect of the alkaloids, but it may be in part due to a depressor reflex originating in pressoreceptor areas, not served by the vagus, and caused by the blood pressure increase in the "recipient dog" outside the perfused leg) Under these conditions, when blood from the "recipient dog" cannot reach the perfused leg, there is no evidence of active vasoconstriction

4 *The rôle of epinephrine* The following facts, observed in the atropinized or vagotomized animal, suggest that epinephrine plays an important rôle in the pressor and cardioaccelerator effect of the veratrum alkaloids the characteristics of the hypertensive response, the apparently humoral transmission by the blood stream of the vasoconstrictor effect, which, in the femoral bed of the dog, cannot be accounted for by the concentration of the veratrum alkaloids present, the characteristic increase in heart rate which cannot be due to a direct action of the veratrum alkaloids upon impulse generation in the heart (section V, E2, p 406)

Direct measurements of epinephrine discharge into the suprarenal vein so far have been made only in a few cases in the dog using the isolated gut of the rabbit to estimate the epinephrine concentration While the available data are insufficient to allow of an accurate quantitative statement, they suggest that at the peak of the blood pressure rise after injection of 1 mgm. protoveratrine into the vagoto-

mized dog, concentrations of epinephrine of the order of 1 100,000 may be reached in the suprarenal vein (154)

The hypertensive effect of the veratrum alkaloids can be obtained by intravenous injections in the spinal cat as well as in intact animals (103, 182) It can be largely abolished by the extirpation of the suprarenal glands Similarly, injection of the alkaloids into the aorta through the celiac artery of the eviscerated cat (154, 182) leads to the characteristic blood pressure and heart rate increase concomitant with contraction of the denervated nictitating membrane and inhibition of uterine activity These effects can be prevented by removal of the suprarenal glands between successive injections It was shown by Kusnetzow (161) that the isolated suprarenal gland perfused with Ringer solution releases epinephrine under the influence of veratrine

The mobilization of epinephrine from the suprarenal gland is a property of the ester alkaloids Cevine, the alkamine of veratridine, for instance, when injected into the aorta through the celiac artery in the spinal cat, proved ineffective in a dose 70 times larger than a strongly effective dose of its veratric acid ester (182)

While a direct effect of the veratrum alkaloids upon the suprarenal medulla leads to a release of epinephrine, the possibility remains that the veratrum alkaloids may act centrally upon sympathetic parts of the autonomic nervous system as well as upon certain parts of its sympathetic outflow, and that the action upon the suprarenal medulla represents one element only in a widespread sympathetic discharge

5 *The possible rôle of the central nervous system* A central vasomotor action was first considered by von Bezold and Hirt (18) when they observed blood pressure increase after veratrine in the vagotomized rabbit In the cat with cold block of the vagus Jarisch and Richter (139) claim to obtain as a rule a blood pressure increase if 0 05 to 0 1 mgm of veratrine hydrochloride is injected intravenously The effect is still obtainable after denervation of the carotid sinus and removal of the thoracic part of the sympathetic outflow of the autonomic nervous system, but is absent or less pronounced after severing the cervical cord, and deepening ether anesthesia also decreases it If a strong central vasomotor stimulation would be caused by large doses of veratridine or protoveratrine, this effect should become apparent in the femoral bed of a "recipient dog" perfused from a "donor dog," when one of the alkaloids is injected into the vagotomized "recipient dog " As mentioned above, under these conditions blood flow remains high in the perfused leg, and there is no evidence of vasoconstrictor impulses being carried from the vasomotor center to the vessels of the perfused leg by way of the femoral and sciatic nerves

While there is no conclusive evidence in favor of a central vasomotor stimulation by large doses of veratrum alkaloids, a few experiments with small doses of veratrine suggest central vasomotor and sympathetic stimulation When the receptor areas in the viscera of the chest are circumvented by injecting veratrine hydrochloride into a vertebral artery of the cat, doses between 0 01 and 0 05 mgm cause a slight increase in blood pressure and acceleration of heart rate (136, 209) The suboccipital injection into the cisterna magna of 0 04 to 0 05 mgm of

veratrine hydrochloride in the rabbit is followed within 3 to 5 minutes by fatal lung edema (141, 209), for which powerful central sympathetic stimulation is considered responsible The rôle of epinephrine in these experiments was not ascertained The phenomenon calls to mind the sudden lung edema in the rabbit caused by intravenous injections of epinephrine (171)

Kunishô (159) observed in rabbits a stimulation of uterine activity after the intravenous injection of 0 001 mgm veratrine per kgm This effect was abolished by sectioning the spinal cord between $C_4$ and $C_5$ After this, doses of 0 1 mgm per kgm were necessary to cause a second increase in uterine activity Both these effects were not influenced by atropine but were entirely prevented by yohimbine. (The direct stimulating effect of veratrine upon the isolated uterus is not modified by yohimbine ) This is consistent with the assumption that epinephrine may be released and act as transmitter as a result of a central stimulating action as well as in consequence of an action upon ganglionic structures like the suprarenal gland. Further observations indicating possible central sympathetic stimulation are the inhibitory effect of small doses of veratrine upon the rabbit intestine in situ which can be prevented by severing the splanchnic nerves (160) and the hyperglycemic action of veratrine in the rabbit, which does not occur after cutting the cervical cord above the origin of the splanchnic nerves (220)

6 *Summary* The veratrum alkaloids in high enough concentrations are vasoconstrictor by direct action upon the vessel wall This is not likely to be of importance in the vasopressor response described above, observed with large doses when the vagus effect upon the heart is excluded The available evidence indicates that epinephrine release plays an important rôle in the vasopressor and cardioaccelerator effect Direct action of the veratrum alkaloids upon the suprarenal medulla causes a discharge of epinephrine. It is possible that in the intact animal a central sympathetic stimulation may also be involved in the effect upon the suprarenal gland The epinephrine releasing action is a property of the ester alkaloids and probably not of the veratrum alkamines

More extensive experimental work with the various pure veratrum alkaloids is required to establish the different sites of their action, especially in the central representation and in the sympathetic outflow of the autonomic nervous system.

E *The Action of the Veratrum Alkaloids upon the Heart.* The elucidation of the chemical structure of the veratrum alkaloids (section I) has increased the interest in their action upon the heart While the veratrum alkamines and cardiac aglycones are similar in their basic steroid structure, they differ in the nature of their additional rings The unsaturated lactone ring at $C_{17}$ of the steroid ring system of the cardiac glycosides appears to be intimately connected with their specific positive inotropic action In place of this structure, veratrum alkamines have a nitrogen-containing ring system This raises the question, as Craig and Jacobs (44) emphasize, "whether the cardiac action of both the cardiac glycosides and veratrine is not a property inherent in the sterol nucleus itself, once given the proper supporting groups in certain positions and the necessary stereochemical configurations "

The major effects of veratrum alkaloids on the heart are a positive inotropic effect, a moderate change in sinus rate, the production of irregularities, and a prolongation of the beat The earliest definitive work in this field already described all but the first of these (18) The more striking cardiodecelerator effects produced by veratrum alkaloids in the intact animal are mediated by the central nervous system and have been discussed in section V, B In the following paragraphs the actions of several veratrum alkaloids on the isolated heart will be discussed insofar as they resemble those of the cardiac glycosides In section VI, A2, E and F the cardiac effects of these substances will be compared with the action of veratrine on nerve and muscle

1 *The positive-inotropic effect* In the isolated heart of a marine snail, *Aplysia limacina*, L , Straub (243,244) found that veratrine increases the maximal tension of the contraction The same effect has been observed in the frog heart (146, 207, 246)

As is the case with other substances possessing positive inotropic action, this property can best be brought out in the hypodynamic heart When this state was induced by perfusion of the frog heart with Ringer solution free of calcium, or by the use of citrate (which removes calcium), Ransom (204) and McCartney and Ransom (180) observed that veratrine caused an increase in the amplitude of the ventricular contraction In the Straub heart veratrine completely abolished the impairment of activity due to calcium deficiency caused by Ringer solution containing $\frac{1}{3}$ of its normal calcium content (207) This positive inotropic action has been confirmed with pure alkaloids in isolated frog hearts perfused from the venous side, using as a measure of activity the restoration of the output to normal when it was decreased by perfusion with Clark solution containing only 50 per cent of the normal calcium content (154, 182) The concentration of protoveratrine or veratridine capable of restoring normal output without changing rate or producing irregularities was of the order of $1 \times 10^{-7}$ while higher concentrations ($2 \times 10^{-4}$) of the alkamine cevine were required

In mammalian hearts the early observations of Hedbom (108) and Kulabko (158) were more recently extended to the specific conduction tissue of dogs' and rabbits' hearts (90, 123, 253, 254) They were also confirmed in experiments on the heart-lung preparation of the dog, on the basis of suspension curves from the right ventricle (88) In each case veratrine produced a marked increase in the amplitude of contraction As pointed out by Krayer and Mendez (152), the positive inotropic effect was not more carefully investigated prior to their own study for two reasons 1, in the intact circulatory system of the mammal, the strong vagal action of veratrum alkaloids entirely obscures the direct cardiac action, and 2, even in the isolated heart the effect is not marked unless the heart shows signs of failure This latter fact was clearly recognized by Hedbom, who stated, "Veratrin und Digitalin sind insofern von Interesse, dass sie zeigen, dass Herzpräparate, welche durch den Einfluss des Physostigmins (und vielleicht auch andere Schädlichkeiten) sehr schlecht arbeiten, durch diese beiden Gifte nochmals recht kräftig stimuliert werden können "

In the absence of a significant cardiac failure, Krayer and Mendez (152) saw

no consistent effect on systemic output, coronary flow, rate, mean arterial pressure, or pulmonary pressure in the heart-lung preparation of the dog with veratrine in doses up to 0.5 mgm There was, however, a distinct though slight decrease in right and left auricular pressure, and an increase in blood supply, effected by raising the level of the blood in the inflow reservoir, was followed by an increase in auricular pressure which was less than that occurring before the administration of veratrine When blood supply was kept constant, a marked decrease of heart volume (occurring without a significant change in heart rate or pulmonary arterial pressure) established clearly the inotropic nature of the veratrine action

Like the cardiac glycosides, veratridine increases the efficiency of the failing mammalian heart while increasing its work performance and its oxygen consumption. The increase in efficiency is especially striking when the diastolic volume of the heart is kept constant (189)

Even severe failure in the isolated heart can be relieved and the work capacity of the heart restored to normal irrespective of whether failure is spontaneous or induced by pharmacological agents (barbituric acid derivatives, local anesthetics, bromine) Under these conditions an effective dose of veratrine causes a marked increase in systemic output, a transient increase in coronary flow, a decrease in auricular pressure, and a very marked reduction in the size of the heart (152)

The interpretation of the change in diastolic volume in the failing heart is complicated by the action of veratrine on pulmonary arterial pressure and stroke volume. While the heart rate decreases usually not more than ten per cent, the stroke volume increases markedly as the total output rises Pulmonary arterial pressure, changes of which have a strong influence upon the diastolic volume of the right ventricle, as a rule increases with the development of failure in the heart-lung preparation This can be due to a change in arterial resistance as a result of alterations in the wall of the pulmonary vessels or it can be due to an elevation of left atrial pressure higher than normal pulmonary arterial pressure when the left ventricle becomes unable to empty Veratrine causes a marked fall of this elevated pulmonary pressure. In a number of experiments with veratrine as well as veratridine (152, 191), the pulmonary arterial pressure increased for up to a minute and then fell markedly Coincident with the rise in the pulmonary pressure, the systemic output rose and the right and left atrial pressures fell Other things being equal, the rise of pulmonary pressure could be accounted for by the increased flow of blood through the lungs resulting from the increased cardiac output. The subsequent fall of pulmonary arterial pressure may be due to the fall of left atrial pressure, that is, to a decrease of back pressure The further possibility remains, however, that a diminution of the resistance of the pulmonary circuit contributes to this fall of pulmonary arterial pressure

The action of veratridine is qualitatively similar to that of veratrine and the pure alkaloid is only slightly more potent than the alkaloid mixture veratrine (191) Cevine, the alkamine of veratridine, still has the characteristic positive inotropic activity but has less than $\frac{1}{\text{?}}$ of the potency of veratridine The minimal effective dose of cevine is 15 to 20 mgm as compared to 0 05 mgm of

veratridine under identical conditions in the heart-lung preparation of the dog with an average total blood volume of about 800 cc The minimal effective dose of protoveratrine in producing the positive inotropic action is about 0 005 mgm. or only $\frac{1}{10}$ of that of veratridine (154) The degree of improvement of a failing heart attainable with protoveratrine, however, is less because of the narrower range between the minimal doses producing positive inotropic effects and those causing irregularities The ratio of minimal irregularity dose to minimal positive inotropic dose is about 13 for veratridine, 5 for veratrine, and not more than 3 for protoveratrine

The duration as well as the magnitude of the positive inotropic effect of veratridine (191) is similar to that of veratrine An initial dose of 0 2 to 0 3 mgm , which in a failing heart restores normal output and normal auricular and pulmonary pressure, has a duration of action of 40 to 100 minutes Smaller doses have a briefer action The maximal effect does not appear abruptly but develops in a period of about five minutes After large doses of cevine, however, the maximal improvement of output in the failing heart appears within one minute of the injection of veratridine (191) Repeated doses of veratrine (152) and veratridine (191) cause a progressively smaller and briefer effect At this stage the effect can still be increased by increasing the dose, and relatively large doses, which given initially would cause irregularities, do not cause disturbances of rhythm but only lead to a transient improvement of output and work capacity The moderate improvement in the work capacity of the failing heart with an initial dose of 0 005 mgm of protoveratrine (154) lasts for about 15 minutes, with an initial dose of 0 015 mgm it lasts for about 30 minutes When subsequent doses of protoveratrine (not causing irregularities) have ceased to have a positive inotropic effect, veratridine is still effective

2 *The effect upon spontaneity and conduction* When the cardiac innervation and the adrenal medulla are excluded from affecting the heart, it is seen that veratrum alkaloids affect the beat of the heart by direct action in manifold ways Bradycardia is usually the earliest effect, but sometimes tachycardia is seen With larger doses, extrasystoles, bigeminy, pulsus alternans, auricular asystole, groups of rapid beats, persistent tachycardia, and ventricular fibrillation may occur Auriculo-ventricular block and various kinds of intraventricular block are also found A correspondingly diverse array of electrocardiographic tracings has been collected (see Boehm, 27, for older literature, 16, 27, 88, 89, 90, 123, 152, 154, 182, 191, 240, 241, 254, 266)

These cardiac irregularities appear to result from two types of changes produced by veratrum alkaloids in cardiac tissue, namely, changes in spontaneity, or tendency to produce spontaneous beats, and impairment of conduction of impulses, probably associated with alteration of the cycle of recovery of excitability after the passage of impulses The diversity of the irregularities which have been reported depends upon the many combinations of site and degree of these effects in the different parts of the heart

In the frog heart isolated according to Straub, the smallest active concentrations of protoveratrine, veratridine, and cevine cause only an increase in the

amplitude of the ventricular contractions With higher concentrations, the heart rate decreases In the isolated mammalian heart (152), initial doses of veratrine which do not produce irregularities have no constant effect on heart rate. In heart failure, especially when caused by barbituric acid derivatives, an initial dose of 0.2 to 0.3 mgm as a rule produces a decrease of heart rate of about 10 per cent Subsequent doses intensify this effect especially in the case of veratrine. Protoveratrine (154) causes no change of heart rate with doses which do not produce irregularities (0 005 to 0 015 mgm ) Even in the minimal positive inotropic dose (15 to 20 mgm ) cevine causes a definite slowing of the heart (191) After large doses of cevine which have caused a marked decrease in rate, veratridine, in doses which, given alone, do not cause a change in rate, produces a transient return of the heart rate to its original level (191)

The spontaneity of parts of the heart other than the sinus node is increased by somewhat higher doses of veratrum alkaloids In inactive strips of mammalian auricle and turtle ventricle veratrine initiates persistent beating (216, 221) Ventricular extrasystoles of various sorts and the dominance of one or more ventricular pacemakers are evidences of an increase in the spontaneity of the ventricles seen in certain stages of the action of veratrum alkaloids No similar increase in spontaneity is seen in veratrinized nerve or skeletal muscle

Impairment of conduction commonly occurs with doses of veratrum alkaloids somewhat greater than those which simply change the sino-auricular spontaneity The sudden appearance of partial A V block in the veratrinized frog heart has been described repeatedly (see Boehm, 27, for references)

Sometimes intraventricular forms of block are the first to occur, leading to abnormalities of the ventricular complexes of the electrocardiogram and sometimes to pulsus alternans (156, 240, 241) Since increased spontaneity and failure of conduction coincide, it is not surprising that ventricular fibrillation often occurs

De Boer (29) stated that veratrine lengthens the refractory period of the frog's ventricle Drury and Love (52), however maintained that de Boer was measuring not excitability but conduction With a more precise method they showed that veratrine may shorten the refractory period of frog ventricle, but that simultaneously conduction may be severely impaired In cardiac conduction tissue, on the other hand, Berk and Wachstein (16) showed a true lengthening of the refractory period The conduction velocity of turtle ventricle, however, undergoes an initial increase before its marked decrease (89) Moreover, the threshold of the frog or turtle ventricle to induction shocks, like that of nerve and muscle (section VI, C) is first lowered and then elevated (48, 89) Hence it is not unlikely that the refractory period may be at an early stage shortened, and later lengthened The data available at present do not illuminate this point

In the isolated frog heart, sufficient doses of protoveratrine veratridine, and cevine cause ventricular extrasystoles and auriculo-ventricular block. With high concentrations, systolic standstill occurs Boehm (26) found this to take place within a few minutes with 1 550 cevadine Systolic standstill is brought about with veratridine by a concentration of $2 \times 10^{-3}$ ($3 \times 10^{-3}$ M) and with cevine by

$1 \times 10^{-2}$M (182) With protoveratrine "Salzberger," Boehm (26) saw only diastolic standstill, whereas in the experiments of Krayer, Moe, and Mendez (154) protoveratrine "Jacobs" in concentrations of $1 \times 10^{-3}$ to $1 \times 10^{-4}$ caused ventricular standstill in systole within 5 minutes

In the heart-lung preparation of the dog, initial doses of 0 5 to 1 0 mgm of veratrine (152) or 0 65 mgm veratridine (191) or repeated smaller doses cause arrhythmias and sudden tachycardia With veratridine the heart rate may remain regular and tachycardia may occur with asystole of the auricle, suggesting A-V nodal or ventricular pacemaker The establishment of permanent tachycardia is often preceded by short periods of acceleration The severest effect is ventricular fibrillation Protoveratrine (154) in doses of 0 02 mgm usually provokes irregularities, auricular asystole and ventricular tachycardia are followed by ventricular fibrillation within 15 minutes after the administration. With large doses of cevine (191), periods of acceleration of the ventricles may occur, but even doses of 70 to 80 mgm do not cause ventricular fibrillation

In producing irregularities, veratridine is considerably more potent than is veratrine (191) For example, under comparable conditions, the administration of as much as 1 5 mgm of veratrine in divided doses over a period of 80 minutes did not produce ventricular fibrillation, whereas this effect was brought about by 0 6 mgm of veratridine given in three equal doses over a period of 50 minutes, and in another experiment by several doses totaling 0 75 mgm given over a period of 90 minutes Protoveratrine (154) is even more potent a cardiac poison than veratridine, their minimal irregularity doses being 0 65 mgm for veratridine, and 0 015 mgm for protoveratrine, a ratio of 43

F *The Complexity of the Circulatory Action of Large Doses of Veratrum Alkaloids in the Intact Animal* The evidence contained in section V makes it understandable that in animals with the autonomic nervous system intact the analysis of the circulatory action of large doses of veratrum alkaloids presents great difficulties The direct influence upon the rate, rhythm, and contractility of the heart, while absent or obscured with small doses, markedly modifies the circulatory action of large doses Again, there are striking differences in this regard between the various pure alkaloids, as is illustrated by the great tendency to cause irregularities of rhythm which makes protoveratrine a substance so much more dangerous than veratridine

It will be impossible, with substances of such varied pharmacological properties as the veratrum alkaloids possess, to analyze completely their circulatory action without a knowledge of the dosage ranges at which the influence upon other organ systems begins to affect the circulatory system and without knowing how this influence manifests itself Reference to this was made when discussing the action upon the respiratory system Relevant evidence for a fruitful discussion is lacking even in the case of the much investigated veratrine response of nerve and muscle

VI The ACTION OF THE VERATRUM ALKALOIDS UPON NERVE AND MUSCLE The striking action of veratrum alkaloids on the skeletal neuromuscular system was first clearly described and analyzed in frog muscle by von Bezold and Hirt

(18) and Prévost (202) It has since been the subject of innumerable researches, most of them with veratrine (the mixture of veratrum alkaloids obtained from the seeds of *Schoenocaulon officinale*)

A. *The Repetitive Response* For many years the opinion prevailed that only muscle was involved in the veratrine response and that veratrine had little or no effect on the nerve. The characteristics of the veratrine effect in muscle were therefore the first to be worked out, and only later was it shown that, except for the mechanical response, most of the phenomena seen in muscle could occur also in nerve.

1 *Skeletal muscle* In order to understand the nature of the veratrine response in nerve and muscle, it is convenient to begin with the classical description of the phenomenon as seen in frog muscle (27) The resting muscle, treated with an appropriate dose of the drug, shows no obvious abnormality If, however, a brief stimulus be delivered to the nerve or muscle, the usual quick contraction of the muscle is followed by a phase of slow relaxation which may last for more than twenty seconds. The myograms resulting from this type of response have varied shapes. Weak veratrinization leads to a twitch like initial contraction and partial relaxation, followed by a slow secondary rise and fall With stronger poisoning, the dip in the myogram diminishes, and the height of the secondary rise grows With even stronger doses the curve presents a smooth rise to a maximum several times the normal twitch height, followed by a long, slow fall to the base line

Since the completely curarized preparation, stimulated directly, gives typical veratrine responses, it may be concluded that the nerve is not necessary for this phenomenon and that the muscle alone is capable of producing it. The same conclusion may be drawn from the fact that denervated muscle exhibits the veratrine response for some time after the nerve has ceased to function (12 74, 203)

It is generally agreed that the veratrine response begins with a twitch much like that of the normal muscle The after-effects, however, have been the subject of much dissension The main point to be settled is whether this part of the response is a tetanus or a contracture. It can be called a tetanus only if it consists of a succession of twitch like responses, each one being conducted along the muscle fiber in which it originated, accompanied by a spike-like electric disturbance If these conducted disturbances are absent, the veratrine response belongs to the heterogeneous group of contractures (83)

Until recent years, the prevailing view was that the slow phase of the veratrine response in muscle is a contracture. This was based on the experiments of all the early investigators purporting to show the absence of electric and mechanical evidence of conducted disturbances during this phase. If the response is to be classified as a tetanus, it must be accompanied by oscillatory electric disturbances, and all the early investigators agreed on the absence of these oscillations.

It is now evident that these experiments bore negative results because the electrical instruments then available were insensitive to the rapid disturbances of low amplitude which may occur in muscle Thus the older investigators

recognized the tetanic nature of some conditions in which the motor unit, containing tens or hundreds of muscle fibers, fired with some degree of synchrony but were unable to detect the finer oscillations associated with the asynchronous firing of single fibers (cf 50) The latter is the mode of the veratrine response insofar as it is a tetanic phenomenon (58) By skillful use of the string galvanometer, Paul Hoffmann (116) was able to demonstrate oscillations in the electromyogram of veratrinized frog muscle, particularly in the early part of the record where the muscular volleys may be more or less synchronized As the degree of veratrinization advanced, the frequency of the oscillations increased and their amplitude decreased With full veratrinization, Hoffmann found a smooth electrogram The string galvanometer records of others resemble more or less those of Hoffmann (28, 101, 143, 203)

With the cathode ray oscillograph, aided by electronic amplifiers, it has been shown conclusively that the veratrine response in muscle is under most circumstances a tetanus This was first demonstrated by Bacq and Brown (12) in normal cat muscles stimulated directly or indirectly and after curarization or denervation With weak or moderate degrees of veratrinization the conducted disturbances demonstrated in the electromyogram account fully for the mechanical record In cats, fowl, frogs, and toads the two-peaked myogram of the weakly veratrinized muscle is accompanied by two corresponding bursts of spikes separated by a relatively silent period (58, 67, 106) In cats, Rosenblueth, Wills and Hoagland (219) observed no element of the mechanical response, even with severe veratrinization, which could not be attributed to the conducted disturbances demonstrated by the electromyogram In frogs and toads, however, Feng (67) and Eichler (58) found, in confirmation of Hoffmann (116), that with severe poisoning the number and duration of oscillations conducted along the muscle fiber decreased despite the maintenance of tension

If the veratrine response is to be classified as a tetanus, it must be propagated from a veratrinized to a non-veratrinized region of the muscle fiber, and hence the latter must show a veratrinic[3] response when the true veratrine response is set up in the veratrinized region The amphibian M sartorius is peculiarly adapted for this experiment because its muscle fibers pass in parallel arrangement almost the full length of the muscle Hence when this muscle is fixed in the middle by gentle pressure and the two ends are made to record mechanically, it is possible to determine whether or not a response set up in one end of the muscle is propagated to the other end If a part of one end of the muscle is poisoned with veratrine (with controls to show the absence of spreading of the alkaloidal mixture along the fibers) and brief stimuli are applied to the poisoned and unpoisoned ends, it will be apparent to what degree the veratrine response is propagated from the poisoned to the unpoisoned end of the muscle

This double myogram experiment was performed in frogs by Biedermann (20) and Riesser and Richter (212) with a clear result The veratrine response was localized in the half of the muscle, part of which had been treated with veratrine In the unpoisoned half, only the initial twitch appeared The older view, that the veratrine response is a contracture, rested on these results

[3] For an explanation of the term veratrinic, see page 433

The same experiment was performed in toads and frogs by Engelmann (61) and many others (65, 66, 67, 87, 121, 124, 179, 267) with the opposite result All of these investigators showed that the veratrine response is under most circumstances, propagated from the poisoned to the unpoisoned area of the sartorius However, with high concentrations of the drug the propagation becomes less and less complete, until the result is not unlike that found by Biedermann (20) Using thermal measurements, and thus avoiding the depression of conduction which may occur at the fixed region of the sartorius in the double myogram, Feng (67) has shown that with moderate veratrinization the propagation from a poisoned to an unpoisoned region may be complete. Hence there could be no question of contracture

Thus the electromyogram and the double myogram agree in the demonstration that the response of veratrinized muscle is largely if not entirely a tetanus Only severe veratrinization of cold blooded muscles produces contracture

Recent experiments of Kuffler (157) on single fibers from the frog's sartorius muscle suggest an explanation for the difference between contracture and tetanus in the veratrine response If a single, brief stimulus is delivered to the veratrinized muscle fiber, a series of action potentials may be recorded from the stimulated spot If, however, the recording electrodes are placed only a few millimeters away it is found that some of the action potentials are absent Some of the impulses originating at the stimulated spot have died out while being conducted along the fiber Thus what is clearly a tetanus when recorded electrically at the stimulated spot in a single fiber would appear as partly tetanus and partly contracture when recorded from the whole muscle with the double myograph "Contracture" may differ from tetanus in the veratrine response only by this failure of conduction The block of conduction produced by large doses of veratrine will be further discussed in section VI, G

2. *Various types of muscle* A veratrine response occurs in all the vertebrate striated muscles in which it has been tested (1, 36, 74, 98 107 125, 147, 219) The phenomenon also occurs in certain invertebrate muscles (210) In other invertebrate muscles the typical myogram is absent Riesser (210) ventures the generalization that the veratrine response occurs in muscles in which there is a tetanogenic system Sereni (235) found cephalopod chromatophores to be a convenient single-cell preparation for the study of the veratrine response

Strong solutions of veratrine have frequently been reported to produce a sustained shortening of excised striated muscle without the intervention of an external stimulus (31, 165, 242) In the hands of Lamm (163) who used the frog sartorius, this phenomenon occurred only with $10^{-2}$ veratrine. Bacq (11), using the rectus abdominis of the frog, found the same result with $10^{-4}$ veratrine

In vertebrate smooth muscle systems veratrine usually affects the rhythm and amplitude of spontaneous mechanical activity when this is present In some instances it produces a slowly developing sustained shortening of the muscle, somewhat similar to what has been described above in skeletal muscle (10, 31, 51, 120, 232) Marfori (176) reported that the response of the frog's bladder to an electric stimulus after veratrine was longer than normal, with a slow relaxation, he considered this to be analogous to the veratrine response in striated

muscle In the nictitating membrance of the cat (218), the phenomenon seen by Marfori occurs on delivering a single brief shock to the nerve supplying the membrane If, however, the membrane is stimulated by epinephrine, veratrine does not increase the response This result indicates that a veratrine response occurs in adrenergic postganglionic nerves but not in this smooth muscle. In view of the fact that veratrum alkaloids cause a release of epinephrine from the adrenal medulla (p 401), it is possible that the abnormalities reported in veratrinized smooth muscle systems are due at least in part to the effects of veratrine on the neuronal elements of these tissues The fact that veratrine sensitizes *isolated intestinal strips from the rabbit or the vas deferens of the guinea* pig to the stimulating effect of potassium ion (11) may have the same explanation At present, however, it is impossible to exclude the existence of a veratrine response in smooth muscles (see also p 401 and p 402)

A number of observers have noted a prolongation of systole in veratrinized cardiac tissue (see Boehm 27, for older literature, 59, 182, 203, 221), occasionally to a marked degree, as in the heart of *Torpedo* (82) This prolonged contraction of cardiac muscle has generally been considered analogous to the veratrine response of skeletal muscle Since the prolongation of contraction in skeletal muscle is due to a tetanic response and since tetanic contraction is generally agreed to be excluded by the properties of cardiac muscle, this interpretation is not warranted today Gilson and Irvine-Jones (89) observed the prolongation of systole in the turtle's ventricle, and attributed it at least in part to the slowing of conduction, which was so marked that relaxation could be seen beginning at the proximal end of the strip while contraction was only beginning at the distal end These effects of veratrum alkaloids on conduction velocity are much more striking in the heart than their effects in nerve and skeletal muscle (section VI, G)

Although the analogy between the contractions of veratrinized auricle or ventricle and those of skeletal muscle has been disappointing, the specialized conduction tissue of the mammalian ventricle has yielded suggestive results. These will be discussed in section VI, F

3 *Nerve* Von Bezold and Hirt (18) considered that veratrine prolonged the state of excitation of nerve in a manner analogous to what they saw in muscle Their evidence, however, was uncertain, and a blast from Fick and Boehm (72) blew this opinion out of the minds of investigators for years

Several attempts are made to test the effect on the muscular twitch of veratrine applied locally to the stimulated region of the motor nerve Most of these were negative In 1902, however, Ioteyko (122) found that some minutes after the application of veratrine to the nerve, a brief stimulus applied anywhere along the nerve produced a typical veratrinic response in the muscle Wible (257, 258) repeated this experiment with the same result and demonstrated by a reversible nerve block between the veratrinized area and the muscle that it was indeed the veratrinized nerve which was producing the veratrinic response of the muscle

These results imply that the stimulated area of veratrinized nerve gives off a

shower of nerve impulses Many investigators had studied the electrogram of veratrinized nerve, however, without finding showers of spike potentials following the usual initial volley Traces of oscillation appeared in the records of Bayliss, Cowan, and Scott (15) and Gasser, Richards, and Grundfest (86)

Clear demonstration of the repetitive response of veratrinized nerve to single brief stimuli was furnished in recent years (7, 53, 68, 70) Feng (68) comments on the probable reasons for previous failures to see this phenomenon

In the amphibian and mammalian peripheral neuromuscular system the most sensitive indicator of the veratrine effect is the muscle (53, 70) With somewhat higher concentrations of the drug in the curarized preparation, repetitive responses may be demonstrated in the ventral root on applying a single shock to the intact nerve trunk, if the nerve is now cut close to the muscle, the repetitive discharges are found to be absent. With a still higher concentration of veratrine, the repetitive discharges arise in the veratrinized nerve trunk itself Thus the intramuscular parts of the nerve fibers, that is, the nerve endings, are more sensitive to this effect of veratrine than the axons in the nerve trunk. The nerve endings are sensitive also to similar effects of guanidine and barium ion (54)

The repetitive response of veratrinized nerve is less striking in frogs than in cats, where the gradually diminishing discharge following a single shock may last almost half a minute and initially involve a large proportion of the nerve fibers in synchronous volleys at a rate as high as 660 per second (7)

The repetitive response to a single brief shock as a result of veratrinization has been observed in A, B, and C fibers of cats, A and C fibers of frogs, and in the nerves of certain invertebrates (7, 15, 53, 60, 86, 218) There is no reason to suppose that sensory axons are not affected as well as motor axons.

4 *Receptors* Whether sensory receptors are subject to the same effect of veratrine as those described for nerve axons and muscle is difficult to judge from the available data. Sneezing and a burning sensation on contact with veratrine are properties of the drug recognized for many years Since the circulatory reflexes (section V, A, B, C) arising as a result of the injection of veratrum alkaloids occur with very small doses, it seems unlikely that the nerve axons are sufficiently poisoned to respond repetitively Yet the afferent discharge on injection of these drugs plays an important rôle in eliciting these reflexes. It is possible that the response of receptors to a given degree of stimulation may be rendered more intense by veratrine, thus producing a more frequent discharge in the afferent nerve.

5 *Synaptic and neuromuscular transmission* The notion that veratrine may act on synaptic and neuromuscular transmission has cropped up repeatedly Lapicque's theory of curarization was based partly on experiments done with veratrine Since the drug can produce repetitive discharges in both nerve and muscle and, with higher doses, can block conduction in both of these tissues (section VI, G), the study of neuromuscular transmission is unusually difficult. No convincing evidence of curarization by veratrine is available

Recent studies indicate that neuromuscular transmission in skeletal muscle is accomplished by the setting up of an adequate "end plate potential" This is

a relative negativity of the end plate region which may best be observed in partially curarized muscles on the arrival of a volley of impulses in the motor nerve When it reaches a critical value, the all-or-none muscular response begins at the region of the end plate and is conducted over the muscle fiber Physostigmine, curare and other drugs known to affect neuromuscular transmission change the size and duration of the end plate potential Veratrine, however, does not significantly change this potential in the frog sartorius, even when repetitive nerve impulses arrive at the neuromuscular junction as a result of the veratrine response of the nerve (157) When an all-or-none response is initiated in the veratrinized muscle fiber at the peak of the end plate potential, its augmented negative afterpotential is merely added to the remnant of the normal end plate potential There is evidence that the potential of the superior cervical ganglion which corresponds to the end plate potential of skeletal muscle is not affected by veratrine (55) Veratrine does not change the sensitivity of skeletal muscles to acetylcholine (37, 187) Thus, although both the nerve and the muscle may show evidences of veratrine response, the process of neuromuscular transmission appears to be unaffected

6 *Summary* Thus veratrine renders certain tissues capable of responding to brief stimuli in a characteristically prolonged manner These responses we designate as veratrine responses Instead of the normal single all-or-none impulse, the tissues exhibit a repetitive response consisting of a series of all-or-none impulses which long outlasts the stimulus The prolonged veratrine response is therefore similar to the afterdischarge observed in some reflex systems

The veratrine response is found in all varieties of nerve and skeletal muscle which have been tested The phenomenon has been observed in the heart only in special circumstances It occurs in a number of non-striated invertebrate muscles, but its occurrence in vertebrate smooth muscle has not been established

B *The Electrogram of Nerve and Muscle* The features of the veratrine response so far considered are those which concern the overall function of nerve and muscle, namely, the origination and propagation of impulses and their mechanical effects It is now appropriate to view some less obvious aspects of this phenomenon in an attempt to discover the mechanism of the abnormal behavior of the tissues involved

1 *Demarcation potential* If a nerve, a muscle, or the heart is injured at one point and a galvanometer is connected from this point to an intact region, the latter is found to be electrically positive to the injured point This difference of potential is called the demarcation potential It is generally agreed that the veratrine response can occur in nerve and muscle without change of the demarcation potential (7, 21, 157), although this potential may rise in one tissue (cat muscle, 219) and fall in another (cat sympathetic ganglion, 218) as a result of treatment with average doses of veratrine High concentrations ($10^{-5}$ and up) of the drug depress the demarcation potential (23, 102, 110), (frog heart, 112) The depression of demarcation potential will be discussed in section VI, G

2 *Spike potential* Using slow galvanometers, the earliest investigators of

the electrogram of veratrinized nerve observed a marked prolongation of the electrical response resulting from a single stimulus (18) With the capillary electrometer, Garten (81) first differentiated two electrical disturbances in the veratrinized olfactory nerve of the pike Both were in the same direction, the intact lead going negative with respect to the crushed lead First came a quick wave similar to that of the nonveratrinized nerve, and this was followed by a slow, prolonged negative wave Hoffmann (116) found the same sequence in veratrinized frog muscle

The first, quick wave of the electrogram of nerve and muscle is commonly referred to as the spike It consistently accompanies the passage of conducted impulses in normal nerve and muscle

Certain doses of veratrine increase the amplitude of the spike potential as seen in the electrogram The increase is slight in nerve axons, but more marked in mammalian muscle, and even more marked in the superior cervical ganglion (7, 97, 218, 219) Its significance is not known Larger doses in each case produce a decrease in spike magnitude Evidence is presented that this decrease of spike magnitude occurs in the absence of block (7), although large doses of veratrine may produce a block of conduction in nerve (section VI, G)

In normal nerve a second maximal spike elicited within a short period after the first has the same amplitude as the first if it occurs any time after the relatively refractory period After veratrine, however, a second maximal spike is smaller than the initial spike if it is delivered even much later than this period If stimuli are applied repetitively at various frequencies, the spike magnitude is seen to reach a minimum 15 to 50 milliseconds after the initial spike After this interval there is a gradual regrowth of spike magnitude, approaching the magnitude of the initial spike at a rate which is an inverse function of the frequency of stimulation (7) The same phenomenon occurs in cat muscle and superior cervical ganglion (218, 219) The time course of this phenomenon is similar to that of the repetitive response and the augmented negative afterpotential This diminution of spike magnitude may be attributed partly to refractoriness of some of the axons as a result of repetitive activity and partly to the presence of a large negative afterpotential Since in cat nerve it is different under anesthesia with different general anesthetics (214) other unknown factors probably also play a rôle

3 *The augmented negative afterpotential* The second wave of the veratrine electrogram is called the negative afterpotential In the electrogram of normal nerve, a brief and relatively minor ripple following the spike is designated the negative afterpotential Graham and Gasser (97) first clearly showed that veratrine produces a tremendous and long lasting negative wave in place of this normal ripple The veratrine afterpotential is not a propagated disturbance similar to the spike, but occurs only in the parts of the nerve or muscle which have been exposed to veratrine An impulse originating in normal nerve, for example, has in this region a normal negative afterpotential, but when passing through a veratrinized region of the nerve, it leaves behind it the local, large, and prolonged negative afterpotential

The augmented negative afterpotential is a constant and striking feature of the veratrine response Its significance will be discussed in section VI, D It has been found in all nerves so far studied (9, 15, 40, 81, 85, 97, 99, 100, 206, 218), in the mammalian sympathetic ganglia (167, 218), in vertebrate skeletal muscle (116, 219) and in the electric organ of *Torpedo* (82) The lack of effect of veratrine on the end plate potential of the skeletal neuromuscular junction and on the corresponding potential of the stellate ganglion has been noted above (p 414)

The magnitude of the augmented negative afterpotential varies with the dose of veratrine and the tissue studied In C nerve fibers it may approach the amplitude of the spike It usually appears to be developing as the spike subsides, coming to a peak of amplitude some milliseconds later In single muscle fibers from the frog's sartorius, Kuffler (157) finds that the interpolation of a spike during the negative afterpotential leads to a temporary annulment of the potential, which then grows again as a result of this second impulse After a single stimulus, the negative afterpotential of nerve may last for seconds or even minutes That of the normal nerve lasts but a few milliseconds

Graham and Gasser (97) found protoveratrine to be less potent than veratrine in producing an augmented negative afterpotential in isolated frog nerve The augmentation of the afterpotential was relatively slight Its duration, however, was greater than that of the negative afterpotential of the veratrinized nerve—how much greater, these authors were unable to say because of the limitations of their recording system

An augmented negative afterpotential may occur in veratrinized frog nerve without the tetanic veratrine response (e g , 97, 157) Clear evidence of the occurrence of a tetanic veratrine response in veratrinized tissues without an augmented negative afterpotential is lacking In single muscle fibers, Kuffler (157) found the augmented negative afterpotential with concentrations of veratrine insufficient to produce a tetanic response to a brief stimulus

Within limits, the negative afterpotentials of successive impulses may be summed This summation is evident when the stimulating electric shock evokes a series of impulses, i e , the tetanic veratrine response The second, third, and several subsequent impulses produce increments of the negative afterpotential begun by the initial impulse There appears to be a limit to the amplitude of negative afterpotential which can be reached by the summation of effects of repeated impulses Thus, beyond a certain frequency of maximal stimulation of a circulated mammalian nerve, no further increase of negative afterpotential occurs Even a rapid rate of stimulation fails to prevent the gradual decline of the negative afterpotential (7) Conversely, the longer the interval between successive stimuli, the more does the electrogram resemble that obtained with the first stimulus In cold-blooded nerve, it may be necessary to wait five minutes after the previous stimulus before a response of equal magnitude may be obtained (81)

The large, prolonged negative afterpotential of veratrinized nerve has usually been considered to be merely an exaggeration of the small, brief negative afterpotential of normal nerve In fact, the occurrence or absence of an augmenta-

tion of a negative wave in the electrogram has been taken as evidence of the presence in various tissues of the basic mechanism underlying "the negative afterpotential" The negative afterpotentials of normal and of veratrinized nerve are sometimes considered to represent a slow phase of the recovery of the demarcation potential after the passage of the depolarization, or spike potential Both are decreased by potassium and by catelectrotonus, which diminish the demarcation potential, and both are increased by calcium and anelectrotonus, which may augment the demarcation potential Both may undergo summation as a result of tetanic stimulation Both are more susceptible than the spike to cold and to anoxia (93, 98, 97, 229) The augmented negative afterpotential of veratrinized nerve differs, however, from that of normal nerve in having an early rising phase. For this reason, it cannot be attributed solely to a slowing of the reparative processes

In view of the fact that the demarcation potential of nerve is maintained by oxidative mechanisms, the relation of the negative afterpotential to respiration is of interest. Schmitt and Gasser (229) found that with a degree of veratrinization which produced an augmented negative afterpotential (but probably no repetitive response), nerve volleys at 2 per second caused a larger increase of oxygen consumption than 120 volleys per second in the nonveratrinized nerve Like that of the normal nerve, the negative afterpotential of the veratrinized nerve is much more sensitive to anoxia than is the spike potential On readmission of oxygen it grows to a greater level than it had before the anoxia began. Thus oxidative metabolism is active during the veratrine negative afterpotential and essential to its maintenance At least in the conditions when no repetitive response occurs, the metabolic cost per nerve impulse is much greater in the veratrinized nerve than in the normal In skeletal muscle, however, when the tetanic veratrine response occurs, the efficiency of the external work performance is the same as that of a normal tetanic response (105) This contrast suggests that the metabolic abnormality of veratrinized tissues is marked in their excitation and conduction systems, and does not significantly involve the contractile system, whose activity accounts for the major part of the metabolism of muscle

4 *Positive afterpotential.* So prominent a feature of the veratrine response is the negative afterpotential that the early positive afterpotential seen in normal nerve, if it still exists, is obscured At the end of the long negative afterpotential of veratrinized nerve, however, there is a positive potential which slowly returns to normal (9, 85, 206) Furthermore, in two ganglionic structures, the superior cervical ganglion of the cat (167, 218) and the retina of the frog (247), at certain stages of veratrinization, one may see an augmented positive afterpotential not long after the end of the spike potential

C *Electrical Excitability* Advancing degrees of veratrinization are generally agreed to produce first a rise and then a fall of resting electrical excitability This has been shown for nerve, muscle, and the heart (7, 18 48, 89, 97, 218) The changes of the parameters of excitation in frog muscle have been variously reported (37, 164) In circulated mammalian nerve the fall of excitability is

accompanied by the same shift of the conventional voltage-capacity curve upwards and to the left as is commonly seen when the excitability of a tissue diminishes, the rheobase rises and the time constant of excitation decreases (7)

In common with certain other conditions to be discussed below in section VI, M, veratrine changes the normal relation of closing anodal and closing cathodal thresholds The anode stimulates at a lower voltage than the cathode, a reversal of the result in normal nerve (7)

The passage of a conducted impulse normally leaves in its wake a characteristic series of changes of electrical excitability Of these, the absolutely refractory period and the succeeding relatively refractory period are, in frog nerve, little affected by veratrine short of doses which block conduction (97) A lengthening of the refractory period has been observed in crustacean nerve (60) (see also section V, E2)

Following the relatively refractory period, there frequently occurs a period of supernormal excitability As a result of treatment with veratrine, this supernormality is often increased in intensity and markedly prolonged

D *Negative Afterpotential, Supernormal Excitability, and Repetitive Response* There has been a tendency to attribute the main effect of veratrine on nerve or muscle to its augmentation of the negative afterpotential and to relegate the supernormality and the repetitive response to secondary positions as consequences of the augmented negative afterpotential A fair degree of correlation has been established in non-veratrinized nerve between the afterpotential and the concurrent electrical excitability (see 63) Positive afterpotential tends to coincide with subnormal excitability, negative afterpotential with supernormal excitability

The fact that veratrine enhances not only the negative afterpotential but also the supernormality has supported the thesis that negativity is a good index of increased excitability The generally accepted fact that sometimes a large and prolonged negative afterpotential occurs in veratrinized nerve in the complete absence of supernormal excitability (7, 97) mars considerably the unqualified acceptability of this thesis In the opinion of Graham and Gasser (97) this fact indicates that some other, unknown factor, in addition to the negative afterpotential, conditions the appearance of supernormality Certainly a large negative afterpotential alone does not warrant the assumption of concurrent supernormality

The repetitive response has been attributed to the excitatory influence of the negative afterpotential, or, in other words, to the supernormal excitability of which the negativity is considered to be the index (86, 157) The occurrence of a spike on the rising phase of the negative afterpotential is taken to be analogous to the occurrence of a spike at the peak of the endplate potential in skeletal muscle, or to the occurrence of a spike after the utilization time at the cathode when electric pulses are applied Thus the negative afterpotential is interpreted as the sign of a local excitatory process which may reach liminal values and produce a conducted disturbance In support of this thesis one may cite the fact that weak veratrinization produces a small increase of negative afterpotential

in single muscle fibers, but no repetition, whereas somewhat stronger veratrinization further increases the negative afterpotential and also produces repetition (157) Of the same import is the general correlation between the amplitude of negative afterpotential and the rate of repetitive response with various doses of veratrine (7), as well as the changes of frequency of repetitive responses at the beginning and toward the end of the negative afterpotential (53, 58, 67, 90)

The unqualified support of this view, too, is impeded by certain discrepancies One is that the repetitive response may occur in the absence of supernormal excitability Another is that with increasingly severe veratrinization the repetitive response may eventually decline while the negative afterpotential continues to grow more intense (7) Furthermore, in frog nerve painted with solutions of veratrine, repetitive responses to single, brief stimuli appear for a short time while the negative afterpotential is not very large, but disappear while the negative afterpotential is steadily growing Finally, on aeration after anoxia the negative afterpotential recovers to a value greater than that of the basal state, while repetitive activity remains depressed (68)

A general correlation certainly exists among these three aspects of the veratrine response namely, negative afterpotential, supernormality and repetitive response The discrepancies noted above, however, indicate that other factors not now understood play a significant rôle The physicochemical basis of the changes discussed here will be considered below in section VI, L

E *The Contractile System of Muscle* The effects of veratrine in nerve and muscle so far discussed are related mainly to the processes of excitation and conduction The prolongation of response to a brief stimulus is common to these two types of tissue, one with and the other without contractile powers In both tissues, this prolonged response is attributed to a tetanic discharge of impulses The other aspects of the veratrine response which will be described below are also common to the two tissues and hence to some extent independent of the contractile system Yet the characteristic evidence of veratrinization has been the peculiar mechanogram of the veratrinized muscle

Skeletal muscle is affected by veratrine in ways which are superficially similar to the positive inotropic action which it has on the heart (section V, E1) Veratrine increases the work done by a skeletal muscle in response to a single stimulus (see Boehm, 27) Yet the data do not permit the interpretation that this effect has the same mechanism in heart and skeletal muscle Thus veratrine increases the work done by a skeletal muscle in response to a single stimulus by causing the response to be a tetanus rather than a single twitch In the heart, the positive inotropic effect cannot be attributed to a tetanic response. Because of the tetanic response of veratrinized skeletal muscle, the comparison of the two tissues is difficult. In the heart, veratrum alkaloids produce an increased efficiency of the beat (189), whereas in skeletal muscle, veratrine does not change the efficiency of tetanic contractions (105) The available data are not sufficient to establish or exclude a positive inotropic effect of veratrine in skeletal muscle

Generally speaking, the features of the veratrine response of skeletal muscle

are fully explained by the known effects of the drug on the excitatory and conduction systems of this tissue

F *Veratrine Response in Conduction Tissue of the Heart* Although the analogy between the contractions of veratrinized auricle or ventricle and skeletal muscle has been disappointing, the specialized conduction tissue of the mammalian ventricle has yielded suggestive results Goldenberg and Rothberger (90) found that in a certain stage of veratrinization, strips of the conduction bundle of the dog's ventricle have electrograms strongly reminiscent of those of veratrinized nerve and muscle The initial negative deflection was followed by a prolonged negative wave The frequency of spontaneous beating was very slow, and the long negative wave was followed by an isoelectric period before the next cycle began Superimposed upon the negative wave in greater or less number were regularly spaced, sharp negative deflections similar in shape to the initial negative deflection but less in amplitude The amplitude of these secondary negative waves increased as the interval from the initial deflection increased The mechanogram showed a large contraction corresponding to the initial deflection, and a variable number of much smaller contractions corresponding to the later members of the series of secondary negative deflections

In analogy with the results in veratrinized nerve and muscle, we may distinguish in these electrograms from specialized conduction tissue of the heart, the initial spike and the augmented and prolonged negative afterpotential as well as the repetitive spikes The mechanogram shows that, although summation of contraction does not occur in the heart, a repetitive contraction occurs following the passage of an initial wave In the increase of amplitude of the successive secondary spikes and contractions, we see the gradual recovery of the spike magnitude and, somewhat later, of the the contractile system The abrupt cessation of secondary beats is similar to what is seen in the single fibers of veratrinized skeletal muscles (58, 67)

The prolonged negative wave of the electrogram of the veratrinized conduction tissue is similar to the negative afterpotential of nerve in being relatively more sensitive to anoxia than the initial and secondary spikes Since with anoxia or cyanide poisoning, the negative afterpotential could be abolished without affecting the secondary spikes and contractions, whereas cooling abolished the secondary spikes and contractions but exaggerated the negative afterpotential, a certain independence between negative afterpotential and repetitive response exists in cardiac conduction tissue as in nerve

When the calcium content of the fluid bathing the veratrinized conduction tissue was increased, the rate at which beats, or groups of beats, occurred was diminished, sometimes to zero, as in nonveratrinized conduction tissue When a beat occurred under these circumstances, it initiated a long series of rapid discharges, characterized by repetitive spikes superimposed upon a long negative afterpotential and by mechanical responses corresponding to the spikes This change in ionic environment seems to accentuate the similarity between the veratrine responses of conduction tissue and the nerve or muscle In veratrin-

ized frog ventricles Seeman and Victoroff (233) found mechanograms and electrograms similar to those described by Goldenberg and Rothberger (90) in veratrinized conduction tissue when the calcium concentration was elevated

In a subsequent paper (91) sodium arsenite was shown to produce responses of cardiac conduction tissue similar to those resulting from veratrinization

G *Conduction, and the Effects of Strong Veratrinization* Weak or moderate veratrinization may slightly accelerate the conduction velocity in the heart (89) The main effect of veratrinization on conduction velocity in nerve as well as the heart, however, is depression, observed with more severe degrees of poisoning When poisoning is severe, this is associated with a deterioration of other phenomena, namely, spike magnitude, negative afterpotential, repetitive activity, electrical excitability and demarcation potential (7, 27, 53, 67, 97)

Conduction is meanwhile blocked in an increasing number of fibers at various distances from the stimulating cathode The probable influence of this factor on the earlier interpretation of the nature of the veratrine response in muscle has been discussed in section VI, A1 For many years the veratrine response of muscle was considered to be a special kind of local contraction, rather than a tetanus, because of experiments in which a failure of conduction of the veratrine component was a critical feature In frog nerve, severely poisoned with veratrine, Graham and Gasser (97) found the negative afterpotential to diminish with distance from the stimulating cathode This they attributed to a block of conduction of fibers at various distances from the cathode It is interesting to note that after complete block of conduction and abolition of the spike by veratrine or other agents in the single axon of the lobster, a large local response still occurs (177)

In excised frog nerve, concentrations of veratrine from 1 200,000 to 1 50,000 produce a decrease in the demarcation potential whose extent is a function of the concentration of the alkaloid mixture When the demarcation potential has fallen to a certain level, conduction fails It may, however, be restored by an atmosphere containing 5 per cent carbon dioxide or by anodal polarization Both of these agencies restore the demarcation potential and at the same time re-establish conduction (169) Under these circumstances, block of conduction would seem to depend upon a critical diminution of demarcation potential Yet in circulated mammalian nerve, block may occur in the absence of a significant change of demarcation potential (7) The circulated nerve is, of course, continuously exposed to a tension of carbon dioxide similar to that exerted by an atmosphere containing 5 per cent of the gas Some other mechanism of block would seem to predominate in these conditions

In the heart the effects of veratrine on conduction are unusually prominent (section V, E2, p 406)

H *Stimulus, Initial Response and Veratrine Response* In summary of the material so far covered it may be said that the effects of veratrine on nerve and skeletal muscle are in most respects the same The effects of veratrine on the heart are in some ways similar to those on nerve and muscle If the dose of

veratrine is not too large, a characteristic group of phenomena occurs when all-or-none responses are set up With larger doses, additional phenomena arise, involving a failure of various features of cellular activity

In moderately veratrinized, resting nerve or skeletal muscle no striking abnormality is evident The oxygen uptake may be unaffected or slightly elevated (183, 229, 242) and the demarcation potential may be slightly raised or lowered High concentrations of veratrine (1 1600 or more) poison certain oxidative systems in mammalian liver brei or slices (17)

Even when all-or-none responses are set up, the initial response is not strikingly different from the normal It consists of a conducted disturbance characterized by a spike potential and, in muscle, a wave of contraction The spike is often somewhat larger than normal, but the absolute and relative refractoriness of the fibers is not significantly changed (94, 97) The electrical excitability as tested with condenser discharges may be slightly elevated The closing anodal threshold is significantly diminished and may be lower than the closing cathodal threshold

Only after the initial response do the striking effects of veratrinization show themselves The initial spike is followed by augmented negative afterpotential, prolonged supernormal excitability, and a long volley of additional spikes, the repetitive response, accompanied in skeletal muscle by tetanic contraction

The veratrine response is therefore probably the result of conditions set up by the initial all-or-none response rather than a direct consequence of the applied stimulus Support for this view might be drawn from the lack of effect of veratrine upon the summation of subliminal stimuli as tested by pairs of shocks (84, 95) Gasser (84) has shown, however, that when tested by single shocks after subliminal tetani, frog nerve shows a prolonged period of elevated excitability despite the absence of preceding all-or-none response To this property of nerve he mainly attributed the enhancement by veratrine of the recruitment of nerve fibers (section VI, K) This effect of subliminal tetani upon excitability is similar to the action of veratrine on the supernormality which follows the spike Nevertheless, the veratrine response occurs in a striking form when a single suprathreshold shock, or at the most two or three shocks, are applied This action of veratrine on excitability therefore does not account for the veratrine response This is borne out by the absence of effect on the veratrine response of electrical stimuli supramaximal with respect to the initial response (7) The veratrine response is a function of all-or-none responses and bears little direct relation to the stimulus

I *The Closing Tetanus and Accommodation* Kodera and Brücke (149) were struck by the analogy between the slow response occurring in weakly veratrinized muscle with long shocks of many times rheobasic strength, and the "closing tetanus" observed in normal frog nerve or muscle with similar stimuli By extrapolating from weak veratrine to no veratrine at all, they proposed that the drug acts on the mechanism responsible for the "closing tetanus," increasing its electrical excitability until the prolonged response becomes the predominant one, even when shocks of the usual strength and duration are used The striking

aspect of the veratrine response, however, is its persistence long after the decay of the brief stimulus which elicited it In contrast to this, the "closing tetanus" lasts little longer than the current which elicited it

An effect of veratrine on the excitable mechanism of nerve responsible for the "closing tetanus" of the indirectly stimulated muscle is apparent from the work of Rosenblueth and del Pozo (217) They studied accommodation in circulated mammalian nerves stimulated by exponentially rising currents. In normal nerve they demonstrated a clear difference between the brief twitches produced by rapidly rising currents and the longer responses to slowly rising currents As the rate of current rise of successive stimuli to the nerve was decreased (the voltage being increased so that the height of submaximal muscle response remained constant), the responses were quick twitches down to a certain rate of current rise, while beyond this rate they were slowly rising and prolonged. At the same point the curve relating the necessary voltage at the various rates of current rise has a break in its continuity Nerve therefore contains two excitable entities, both involving conducted disturbances, one of which accommodates quickly and is associated with a brief twitch of the muscle, while the other accommodates slowly and is associated with a tetanic discharge lasting throughout the application of current. There can be little doubt that the latter, here studied in relation to slowly rising currents, is the excitability of the "closing tetanus " when studied with direct current pulses (see also 238)

Veratrine was found to change the accommodation of nerve chiefly with respect to this latter component, so that with a given rate of rise much less peak voltage was necessary in order to produce a prolonged response (217)

In veratrinized muscle, as in nerve, a slowly accommodating response with relatively low threshold occurs During the passage of a relatively weak constant current through veratrinized frog muscle, Meirowsky (181) observed a mechanical response with wave-like variations in intensity

Since the prolonged veratrine response can occur on stimulation with brief shocks no stronger than those required to elicit single impulses in normal nerves and muscles, it is impossible to attribute the repetitive response to a failure to accommodate to a steady stimulus applied from outside Conceivably, the augmented negative afterpotential resulting from the initial impulse might have the effect of an electrical current applied from outside Against this possibility stands the relatively slight change in the excitability of the slowly accommodating mechanism (68 217) Moreover, in the single muscle fiber, each repetitive spike briefly annuls the negative afterpotential of the previous spike before its own negative afterpotential begins to grow (157) Hence the negativity appears to be interrupted rather than sustained in the single fiber, and a reactivation seems more likely than a failure of accommodation For these reasons it seems improbable that the change of accommodation produced by veratrine contributes to the production of the repetitive response

J *Factors Affecting the Veratrine Response* Whatever they may be, the results of all-or none response which are so strikingly affected by veratrine are less stable than the all-or-none response itself Summation, fatigue, and the

influence of environmental changes affect the veratrine response to a greater degree than the all-or-none response

1 *Summation* Kodera and Brücke (149), confirmed by Hou (117), observed that weakly veratrinized frog muscle, when curarized, had two distinct excitability curves, depending on what type of response was taken as an end point With stimuli of a certain duration applied successively at increasing strengths, the first response to be seen was a simple twitch as in the normal Only when the strength was much greater did the slow veratrine response appear Strength-duration curves plotted in the conventional manner for the veratrine response lay above and to the right of those constructed with the twitch as an end point Indeed, the rheobase of the veratrine response might be as much as 100 times that of the twitch and the time constant of excitability of the former 20 times that of the latter As the concentration of veratrine applied to the muscle was increased, there was little change in the twitch excitability, but that of the veratrine response increased with respect to strength as well as duration until the two excitabilities were indistinguishable, i e , any effective shock elicited a veratrine response

A very similar dichotomy of excitabilities was described by Bremer (33) in connection with his "neuromuscular contracture," namely, that single twitches occurred with shocks of relatively short duration, while long shocks produced veratrinic responses (although no veratrine was present) The necessary condition of Bremer's veratrinic "neuromuscular contracture" is stimulation of the frog nerve by two shocks separated by a brief interval Since strong shocks of sufficient duration are known to set up repetitive firing in frog nerves at a sufficiently rapid rate, it seems likely that the prolonged responses of Kodera and Brücke (149) have the same initiating mechanism as Bremer's phenomenon It has many times been demonstrated that with weak veratrinization a pair of stimuli delivered at a short interval may elicit a typical veratrine response, when one stimulus produces only a brief twitch Thus the excitability curves plotted by Kodera and Brucke (149) for veratrine responses may represent the strength and duration of shocks which produce repetitive response in the nerve The rôle of veratrine would then be to render the nerve self-reactivating When the veratrinization is weak, a summation of the effects of two maximal stimuli may be necessary to demonstrate the self-reactivation

2 *Fatigue* When a single all-or-none response is elicited from a moderately veratrinized nerve or muscle, the augmented negative afterpotential immediately grows, supernormal excitability is demonstrable and the volley of repetitive all-or-none responses follows, each additional response adding its veratrine effects to the previous ones Whereas in the early part of this complex veratrine response a summation is apparent from a still-growing negative afterpotential and an acceleration of the repetitive spikes, very soon the response passes a maximum and gradually wanes In single fibers (58, 67) and in the conduction tissue of the heart (90), the rate of repetitive activity diminishes slightly and then suddenly ceases In multi-fibered nerves or muscles, the waning of the veratrine response may last for thirty seconds or more, and full recovery may not occur for as long

as five minutes Thus the veratrine response, while at the beginning seemingly self perpetuating, is eventually self limiting

If additional stimuli are applied before recovery is complete, the resulting initial response is normal soon after the relatively refractory period, although for some time spike magnitude may be decreased (section VI, B2) The veratrine response is, however, more or less intensified

For stimuli applied after the maximum of the veratrine response, the later they are applied, the greater is the intensification of the veratrine response If stimuli are applied regularly at suitable intervals, this intensification is progressively lessened Thus the veratrine component of the response to each successive shock is diminished, until, at moderate rates of stimulation it may after a few shocks entirely disappear The normal all-or none spike or twitch remains, without augmented negative afterpotential, supernormality, or repetitive response Thus, while the all-or-none responses to electrical stimuli remain normal, the veratrine component has fatigued

3 *Other Factors* An instability of the veratrine response as compared to that of the all-or none response is observed also when the physical or chemical environment of the tissue is changed The negative afterpotential, the mechanical veratrine response of muscle, and the repetitive response of nerve are more sensitive than the spike or twitch to cold, to anoxia (68, 97, 162, 187), and to carbon monoxide (229) The recovery of negative afterpotential after cold is slower than that of the spike, and after anoxia or during illumination of nerves treated with carbon monoxide, the negative afterpotential, unlike the spike, rises temporarily to a higher level than before the poisoning

Changes in the common inorganic ions affect the veratrine response to a greater degree than the all-or-none response A comparison of the effects of veratrine with those of the common inorganic ions shows that the effects of veratrine cannot be due to a simple imitation or exaggeration of the effects of any one of these Yet there are important relations between some of these ions and veratrine For example, the effects of veratrine are sensitive to changes in calcium concentration of the perfusing or bathing fluid When lack of calcium has weakened the beat of the frog heart, veratrum alkaloids restore the beat to normal (154, 180, 182, 204, 207) (section V, E1) On the other hand, lack of calcium favors the production of cardiac irregularities by veratrine (207) Thus in the heart with respect to contraction, calcium and veratrine are synergists, but with respect to excitation and conduction, they are antagonists

In nerve and muscle the antagonism of calcium to most of the effects of veratrine is marked The dose of veratrine necessary to produce a minimal veratrine response in excised frog muscle is a continuous function of the calcium content of the bathing fluid A change of calcium chloride content from 0 1 to 0.5 per cent multiplies by ten the minimal dose of veratrine (53, 68, 163, 187)

This antagonism recalls the generalization that calcium stabilizes biological membranes Calcium lack may be associated with spontaneous discharge of nerve or muscle impulses and a marked diminution of accommodation With veratrine, spontaneous discharge is absent and the accommodation is

markedly changed Lack of calcium does, however, sometimes lead to responses somewhat reminiscent of the veratrine response (162) Calcium opposes the diminution of demarcation potential produced by veratrine or potassium (102) It also opposes the excitatory effect of potassium in frog muscle when this is favored by veratrine (11, 107) Calcium counteracts the paralyzing action of strong concentrations of veratrine on the frog nerve-muscle preparation (65) Citing experiments on dogs Bacq and Goffart (14) advocated the use of calcium salts together with magnesium salts for combatting the toxic effects of veratrum alkaloids

In some respects the antagonism of calcium to veratrine in nerve is replaced by a similarity of effects A high calcium content of the bathing fluid favors the appearance of recruitment in nerve fibers (84, 255) High calcium concentration also enhances the negative afterpotential and lengthens the supernormal period of frog nerve (93) Veratrine produces these same effects, but to a greater degree (section VI, B3 and C)

The relations of veratrine with potassium are as tangled as those with calcium The dose of veratrine necessary to produce a minimal veratrine response on electrical stimulation in frog muscle increases with the potassium content of the bathing fluid (187, 248, see also Boehm, 27) Potassium also diminishes the negative afterpotential and repetitive activity of the conduction tissue of the dog's heart (90) On the other hand, veratrine sensitizes smooth and striated muscles, nerve, and probably also certain receptors to the stimulating action of potassium (8, 11, 107, 188, 215, 237) With respect to this action in muscle calcium antagonizes the effects of veratrine, i e , desensitizes the muscle to potassium The theoretical importance of the sensitization to potassium will be discussed in section VI, L

Magnesium and strontium, like calcium, oppose the effects of veratrine (11, 102, 134, 184, 185, 186, 248) Barium, however, may cause repetitive responses somewhat similar to veratrine responses (54, 70, 91, 248), and veratrine sensitizes certain responses to barium and to rubidium, as to potassium (8, 11)

Quinine is antagonistic to veratrine in several ways It decreases the mechanical responses and the repetitive spikes of veratrinized muscle When larger doses of veratrine are used, quinine opposes the block of conduction which ensues Whereas stimulation of non-veratrinized muscle by potassium is relatively unaffected by quinine, the sensitization of muscle to potassium by veratrine is impeded by quinine (106, 187, 188, 248) In these relations to veratrine it resembles calcium

Quinine possesses a rather general action against repetitive responses While enhancing twitch tension and the spike potential in normal muscle, it diminishes the tension of tetani and the corresponding spikes The repetitive responses of physostigminized muscle stimulated indirectly are abolished (106) Fibrillation in the heart is opposed Several muscular phenomena similar to the veratrine response are also antagonized by quinine, namely, Bremer's "neuromuscular contracture" (34), myotonia of man and goats (144, 150), and the veratrinic responses to 9-phenanthroate and dihydronaphthacridine carbonic acid (3) (section VI, M)

A diverse group of drugs which has been reported to antagonize veratrine effects in muscle need only be listed cholesterol (173), atropine (107, 168, 211, 248), pilocarpine (168), santonin (248), camphor (231, 250), cocaine and procaine (211, 231), salicylate, and tyrosine ester (230) According to Machens (173) lecithin enhances the action of veratrine in muscle

K. *Interaction, Recruitment, and the Two Peaked Myogram* The responses of nerve and muscle fibers after conduction are normally all-or none in character, and there is valid evidence that each fiber is normally insulated from its neighbor in nerve trunks and muscles, so that each impulse passes to the end of its individual fiber without stimulating other fibers Accordingly the height of contraction of a muscle in a single twitch has been generally considered to be rather strictly a function of the number of fibers stimulated The same laws cannot be applied to the veratrine response of frog muscle (18, 162, 192) With stimuli of increasing strength, from threshold to maximal, applied to curarized and veratrinized muscle, the rate of increase of the early, twitch like part of the mechanogram is much like the normal, but that of the late, prolonged part follows a different course Thus the response to a slightly supraliminal stimulus consists of a relatively marked twitch like phase and hardly any prolonged contraction, but with increasingly stronger stimuli the proportion between the prolonged contraction and the twitch grows steadily If we take the height of the twitch like phase to represent the number of fibers stimulated, then the amount of prolonged contraction per stimulated fiber increases with the number of fibers stimulated (see also Querido, 203)

In 1931, Wyss (265) suggested that under the influence of veratrine the impulses in muscle fibers are transmitted to adjacent fibers This mechanism would explain the phenomenon described above if the excitation of neighboring fibers is subject to summation In this case a few scattered fibers activated by the stimulus would produce little effect on unstimulated fibers and hence little after effect, but with a greater density of initially active fibers the effect on neighboring fibers would be sufficient to excite some of these These additional impulses would augment the excitatory influence of the initially active group on other fibers not yet rendered active Herman (111) tested this theory by eliciting twitches of identical submaximal height in veratrinized muscle with stimuli from localized and from diffuse electrodes. The twitch from diffuse electrodes was followed by a longer lasting and greater veratrine component than that from localized electrodes If all the fibers within a certain radius from the localized cathode were excited in the latter case, only a few fibers beyond the periphery of this field would be available for the excitatory effect of adjacent active fibers If, on the other hand an adequate density of initially active fibers were interspersed with inactive ones the conditions would be favorable for stimulation of the inactive fibers by adjacent, active ones

Whereas the theory of interaction among muscle fibers remains in this speculative plane, interaction among nerve axons is supported by clear experimental results. Feng and Li (69) showed that veratrine may cause the activity of some fibers of a nerve trunk to initiate activity in adjacent fibers. They stimulated one ventral root (A) and recorded from another (B), both having been cut cen-

trally and the nerve trunk into which they both led having been cut peripherally Without veratrine, stimulation at A produced no response from the fibers of B, application of veratrine to the common nerve trunk led to the appearance of impulses at B, 4 to 30 milliseconds after stimulation at A

Veratrine enhances and prolongs changes favoring interaction among fibers observed in non-veratrinized nerve (214) If the excitability of a zone of normal nerve trunk is raised by one of several methods, the passage of impulses along some of these fibers induces activity in other fibers at this zone The new impulses arise either during the rising phase of the spike potential, or after the spike potential (see 178, 214, for references) If veratrine is present, the passage of impulses in the conditioning fibers induces such marked excitation in the test fibers that new impulses arise in the latter

A phenomenon resembling interaction, which is similarly enhanced by veratrine, is what Gasser (84) designated as recruitment of nerve fibers If a nerve trunk is stimulated with submaximal electric shocks at a relatively high frequency, the number of nerve fibers responding to the stimuli gradually increases as more and more previously inactive fibers are recruited Factors which favor the negative afterpotential also favor this phenomenon After veratrinization, a remarkable degree of recruitment occurs with notably lower frequencies of stimulation than in normal nerve Gasser attributed this mainly to a lengthened period of summation of subliminal stimuli, which he demonstrated to be present by testing the excitability of the nerve after a subliminal tetanus Rosenblueth (215), however, showed that recruitment occurs independently of the applied electrical stimuli He therefore attributed recruitment mainly to the influence of responses in the lower-threshold fibers on their not-yet-recruited neighbors, that is, to interaction among nerve fibers

The effect of veratrine upon the interaction among fibers, if it applies as well to muscle fibers as it does to nerve fibers, suggests an explanation for the two-peaked mechanogram of veratrinized frog muscle When the dose of veratrine is relatively small, a single maximal stimulus to the nerve or muscle elicits a twitch-like initial contraction and partial relaxation, followed by a slow secondary rise and fall (see Boehm, 27) The electrogram of a multifibered muscle in this condition shows a pause between the synchronous initial spike and the repetitive discharge of some of the fibers (58)

If only a few fibers are sufficiently veratrinized to respond repetitively when a single impulse is elicited in them, the first repetitive response of these may be expected to add its excitatory effect to whatever subliminal excitatory state remains in the fibers which did not respond repetitively Some of the latter may be expected to respond to this summation with all-or-none responses, and the excitatory effects of these would affect still other previously non-repetitive fibers Thus by the interaction and summation peculiar to veratrinized tissue, a progressively greater fraction of the muscle would become involved in an intense veratrine response Hence the original maximal twitch would give way, first to a partial relaxation because at the beginning only a few fibers fire repetitively, but then to a progressive increase of contraction as more and more fibers are recruited

The repetitive response so characteristic of veratrinized nerve and muscle may result from the same conditions that render the interaction among nerve fibers so prominent. Since responses in some fibers of a veratrinized nerve trunk can so markedly affect other fibers as to elicit new responses from them, the setting up by the initial response of additional responses in the same fiber should also occur. For this it is necessary that the excitatory state resulting from the initial response should outlast the refractory period, and should be capable of acting on the same fiber at least as readily as it does on neighboring fibers. Since even in non veratrinized nerve a slight excitatory interaction is present which outlasts the refractory period of the conditioning fiber, the first of these conditions is probably satisfied. The distance of even the closest neighboring fibers should attenuate the excitatory influence, and hence the conditioning fiber itself, if susceptible, should be more strongly affected than its neighbors.

Since the possibility exists that repetition and interaction depend on the same excitatory effect of the initial response in veratrinized nerve and muscle, any clue to the mechanism of interaction may provide solutions to many aspects of the veratrine problem.

The rôle of the electric potentials of the conditioning fibers in causing excitation of their neighboring fibers is difficult to analyze in veratrinized nerve. Recruitment and interaction occur in nerves which show more or less repetitive responses to single shocks and more or less of the augmented negative afterpotential. It is generally agreed that in non veratrinized nerve the electrotonic effects of the spike potential of some fibers on their neighbors is responsible for a rise and a subsequent fall of excitability of the latter. The initial spike has the same effect in veratrinized nerve (215). In the repetitive response, however, many spikes occur in the active fibers asynchronously for a long period. That the excitatory state set up by these spikes in neighboring fibers may play some rôle in interaction is suggested by the fact that interaction is poor when a diminution of spike amplitude accompanies the veratrine response (214). A series of spikes in one fiber might be expected to have the same effect on a nearby fiber as a subliminal tetanus, and hence lead in the veratrinized nerve to the long period of increased excitability which Gasser (84) detected in these conditions.

The explanation of the late phase of interaction in non veratrinized nerves is controversial. Marrazzi and Lorente de Nó (178) attributed it to prolonged electrotonic effects similar to those briefly described by Lorente de Nó and Davis (170) after the application of direct current. In the veratrinized nerve, the abnormal development of the negative afterpotential contributes to the difficulties of analysis of interaction. Gasser (84) noted that the factors known to augment the negative afterpotential—among them, veratrine being the most prominent—favored the appearance of the phenomenon of recruitment. But interaction in veratrinized nerve may be poor when the negative afterpotential is pronounced, and vice versa, it may be pronounced when the negative after potential is only slightly greater than that of normal nerve. Furthermore, in non veratrinized nerve the late phase of interaction does not follow the course of the afterpotentials and is as great as that occurring during the spike, despite the small amplitude of the afterpotentials in these conditions (214). These

discrepancies impelled Rosenblueth (214) to dismiss the theory that the late phase of interaction depends upon changes of the inactive fibers resulting from the electrical potentials of the active fibers He proposed that the late interaction is due to a chemical substance, released from the active fibers as the impulses pass by and diffusing to the inactive fibers, there to act in an excitatory manner

In another field, that of neuro-effector transmission, considerable advance resulted when Otto Loewi conceived the simple but effective experiment of determining the effect of the products of stimulation of the nerves of a donor heart by perfusing the fluid from this heart through a test heart Perhaps the same contribution to the problem of interaction between veratrinized fibers has been made by Szent-Györgyi, Bacq, and Goffart (245) They perfused the veratrinized hind legs of a frog and led the perfusate through the veratrinized hind legs of another frog When the muscles of the donor frog were stimulated, they responded in the typical veratrine manner And when the fluid which was perfusing the donor preparation during its veratrine response reached the test preparation the latter also showed muscular contraction Curarization did not interfere with the phenomenon Miyake (188) has confirmed these results Hence activity of the veratrinized donor muscles imparted to the perfusion fluid the power of exciting activity in the veratrinized test muscles Here the electrical field of the donor muscles cannot act on the test muscles The exciting influence, whether released into the perfusing fluid from the active muscles, or by their activity unmasked, must be a chemical change carried by the perfusion stream And since chemical interaction is effective in this way between distant muscle fibers, it can also be effective between nearby fibers

L *Theories of the Veratrine Response* Enough has been stated to make it plain that the veratrine response is a result of conditions set up by the initial all-or-none response of muscle and nerve The literature as to the theory of the veratrine effect is devoted mainly to the nature of these conditions

The theories that the veratrine effect is due to some increase of or sensitization to the potentials developed in nerve and muscle as a result of the all-or-none response have been discussed in section VI, D and K Veratrine does affect markedly the negative after-potentials in nerve and muscle and also increases the excitability of these elements to electric current Despite the discrepant experimental results listed in section VI, D, these electrical theories cannot at present be dismissed They do not attempt, however, to analyze the physicochemical mechanism by which veratrine renders abnormal these potentials or this excitability

The chemical theories of the veratrine response suppose that all-or-none responses of veratrinized nerves or muscles cause chemical changes in the cellular environment which are responsible for the peculiarities of the veratrine response One such theory is that of von Frey (78) and Lamm (162), who proposed that veratrine is itself changed as a result of the all-or-none response of muscle into a substance which produces further contraction In support of this view Lamm cited two experiments In one, he mixed a quantity of veratrine with chopped muscle and then extracted the muscle with ether When he redissolved the

ether extract and assayed it on the frog sartorius, he recovered more veratrine activity than was accounted for by the quantity of veratrine originally added to the muscle. The result of this experiment needs confirmation using pure veratrum alkaloids In the other experiment, Lamm immersed a succession of muscles in Ringer's solution containing originally a certain concentration of veratrine and measured for each muscle the time of immersion before a minimal veratrine response occurred on electrical stimulation The second, third, and fourth muscles required successively briefer immersion before the veratrine effect was evident. The later muscles of the series required successively longer immersion until finally no veratrine response could be elicited Lamm's interpretation of these results was that contact of the veratrine with muscles changed the veratrine into a more potent form, at the same time fixing some of it in the muscle substance

Verzár and Felter (252) could not accept the theory of von Frey and Lamm that veratrine was chemically changed by the muscle because so many substances of diverse chemical nature have the same effect as veratrine As an alternative, they suggested that veratrine and these other substances render the muscle more permeable to a stimulating substance released as a result of the twitch They were aware of the supernormal period of non veratrinized tissues and were actually proposing that supernormality is the result of this stimulating substance Fatigue of the veratrine effect would occur, according to their view, when the stimulating substance has penetrated into the muscle cells They had nothing to say about the chemical nature of this stimulating substance

The experiment of Szent-Györgyi, Bacq and Goffart (245) described on p 430 suggested the possibility of identifying a stimulating substance released from muscle during the veratrine response Since the stimulating substance has its effect on the test frog muscles even after complete curarization (13), it cannot be acetylcholine, whose stimulating effect on muscle is abolished by curare Moreover, acetylcholine from the test muscle would be largely destroyed in the course of the perfusion by the tissue cholinesterase. In certain concentrations veratridine and veratrine have anti-cholinesterase activity (9, 193) Veratrine does not, however, change the threshold of muscle to acetylcholine (37, 187)

Since the responses of the test muscles resembled those seen when the potassium ion content of the perfusion fluid is elevated, the behavior of potassium was investigated (13) It was found that the potassium content of the perfusion fluid coming from the donor muscles during muscular activity was enough to stimulate the muscles of the veratrinized test frog If fresh perfusion fluid whose potassium content had been elevated to the level found in the perfusate of the stimulated donor muscles was perfused through veratrinized test muscles, responses were produced in the latter which matched those seen in the experiment of Szent-Györgyi, Bacq, and Goffart (245) Veratrinization of the donor muscles increased only slightly the potassium content of the perfusate from unstimulated or stimulated muscles The main rôle of veratrine was the sensitization of the test muscles to potassium (section VI, J3) Thus tetanic muscular activity

can release enough potassium ion to stimulate a distant veratrinized muscle even after dilution in the perfusion fluid

On the basis of these results Bacq (11) proposed that potassium ion is the stimulating substance released by the all-or-none response of veratrinized muscle which accounts for the veratrine response When applied to muscle in high enough concentration, potassium ion elicits all-or-none responses Denervation and curarization do not interfere with these effects During contraction muscle loses some of its abundant intracellular potassium in exchange for sodium (71). Bacq supposed that the potassium liberated at the surface of the normal muscle cell as a result of the twitch is quickly rendered incapable of restimulating the muscle cell Veratrine would then impede the inactivation of potassium and hence permit it to produce a second response from the muscle This response would of course release more potassium, which would then intensify the restimulating effect Thus veratrine would render the muscle self-reactivating by making the potassium released with the initial twitch an effective stimulating agent

In investigating the mechanism of the late phase of interaction among nerve fibers, Rosenblueth (214) considered the possibility that potassium released from the conditioning fiber might change the excitability of the tested fiber Potassium ion injected into the circulation of the nerve favors interaction This action is accentuated by veratrine, and veratrine sensitizes the nerve to this action of potassium Rosenblueth therefore tentatively proposed that the late phase of interaction between nerve fibers is due to the diffusion of potassium from the active fibers to nearby inactive ones The enhancement of interaction by veratrine might then be due partly to the repetitive activity of the conditioning fibers and partly to the sensitization of the test fibers to potassium

The potassium theory of the veratrine response should be considered at present as no more than a working hypothesis According to the argument of Bacq (11) it would account for repetitive response to single, brief stimuli Rosenblueth's (214) experiments make it a plausible explanation of interaction Yet recruitment, a phenomenon closely related to interaction in at least some respects, is opposed by potassium and enhanced by calcium (84, 255) Moreover, potassium in concentrations near that of physiological fluids opposes the action of veratrine Since potassium decreases the demarcation potential, it is conceivable that potassium remaining in relatively high concentrations outside the nerve, as supposed in Bacq's theory, might account for the negative after potential The depressing effect of potassium on the spike potential might also account for the diminution of spike amplitude observed after the initial spike in the veratrine response Yet increasing the potassium content of the bathing fluid decreases the negative afterpotential of non-veratrinized nerve (93) The potassium theory also fails to account for the increased metabolism of the period of afterpotentials demonstrated in nerve by Schmitt and Gasser (229) Guanidine and aconitine also sensitize muscle to potassium (11, 107), yet their action is not identical with that of veratrine

M *Veratrinic Responses* The effects of veratrine on the frog's gastrocnemius

are so striking and so easy to demonstrate that the rôle of this particular mixture of alkaloids in bringing about the characteristic response is apt to be over emphasized There are many other ways of producing responses similar to the veratrine response. Since responses which resemble those produced by muscarine or nicotine are called muscarinic or nicotinic, it seems proper to designate those which resemble the veratrine response as veratrinic

Most of the veratrinic responses have been less completely analyzed than the veratrine response itself Hence the concepts developed for the latter have been applied to the former In general these veratrinic responses have been interpreted as contractures on the basis of the same sort of evidence which led to the opinion that the veratrine response is a contracture In a few instances this interpretation has been revised on the same basis which led to the revision of the opinion on the veratrine response. In others the crucial experiments have not been performed

A group of veratrinic responses has been shown to occur in the absence of any pharmacological agent. Occasionally otherwise normal amphibian muscles have been reported to respond to single slightly supramaximal induction shocks with a veratrinic myogram (31) which, like the veratrine myogram itself, diminishes with repeated stimulation until it resembles the normal twitch The variables which favor the appearance of this phenomenon in some frogs and not in others are little understood

Bremer (33) has studied another such phenomenon, which he called "neuromuscular contracture" It occurs in a certain proportion of frogs or toads The critical circumstance for its production is the arrival at the muscle, at an interval of about 3 milliseconds, of two stimuli The veratrinic response occurs on stimulation via the nerve or on direct stimulation of the normal or curarized muscle Bremer was unable to find oscillatory action potentials accompanying the response and hence called the phenomenon a contracture His apparatus, however, was too insensitive to record fibrillary activity of the muscle The rôle of summation and fatigue in Bremer's phenomenon is similar to its rôle in the veratrine response Quinine opposes Bremer's phenomenon, as it does the veratrine response (34)

Eccles and O'Connor (56) observed repetitive responses of a few of the fibers of cat muscles stimulated with single nerve volleys They were able to localize the discharges to the muscles rather than the nerves Two nerve volleys at a brief interval produced repetitive responses in a greater fraction of the fibers

Tiegel's contracture has often been likened to the veratrine response (22) It consists of a slow relaxation after the twitch produced by a very strong but brief single electrical stimulus It is generally considered to be a contracture due to the direct effect of the strong stimulating current on the contractile mechanism at the cathode. The similarities to the veratrine response are not so striking as in the case of Bremer's phenomenon (83) In view of the change in the status of the veratrine response, however, it seems desirable to reinvestigate with modern techniques the possibility that the response called Tiegel's contracture may be propagated

In the clinical syndrome called myotonia the muscular response is strikingly like the veratrine response Myotonia refers to the muscular responses occurring in persons with the familial diseases, Myotonia Congenita (Thomsen's disease) and Atrophia Myotonica (Curschmann's disease) (205) If these persons move slowly, their muscular contractions are normal Sudden or strong muscular efforts, however, lead to prolonged contraction of the muscles involved, or, from the point of view of the patient, to the inability to relax the muscles for a period of many seconds After repeated efforts without rest, relaxation gradually returns, and normal control of the muscles is possible Thus with respect to summation and fatigue, myotonic responses are much like veratrine responses The myotonic response is accompanied by a repetitive firing of the individual muscle fibers, even when it is set up by single volleys in the motor nerve In the hereditary myotonia of goats, which is otherwise similar to the disease of man, the myotonic response occurs, with repetitive discharge of the muscle, even after complete curarization and in denervated muscles (35) That this muscular phase of myotonia does not explain the whole syndrome in man is apparent from the work of Denny-Brown and Nevin (49) Another locus of abnormally prolonged activity must be invoked, but it is not clear whether receptors, axons, or the central nervous system are the site of this abnormality

The myotonic muscle of man and goat gives unusually prolonged and vigorous responses on mechanical stimulation This, indeed, might be expected from muscles which respond in this manner to stimulation via the motor nerves But it has not been clearly demonstrated that there is actually an increase of mechanical excitability, i e , that less force is necessary to set off all-or-none responses in these muscles

In myotonic muscles, as in veratrinized muscles, there is a relatively low anodal closing threshold, which may be lower than the cathodal closing threshold That the mechanism of accommodation of muscle is affected in myotonia as in veratrinization is suggested by the slow contractions obtained with direct current (62)

Myotonic responses are like veratrine responses in being antagonized by calcium and by quinine (144, 205) In myotonic goats, the muscles are stimulated by intra-arterial doses of potassium chloride one-tenth as great as those which are necessary in the muscles of normal goats (35)

A second group of veratrinic responses may be produced by treating muscles with certain pharmacological agents Glycerin and other agents which dehydrate frog muscle may produce a veratrinic response (228) Several aldehydes, including formaldehyde, and a number of inorganic ions, including perchlorate, have been shown to do the same (145, 252) These effects of glycerin and formaldehyde are antagonized by quinine (187) Certain derivatives of colchicine have been reported to have a veratrinic effect (80, 126)

Tetraethyl ammonium ion causes responses in frog and mammalian nerves which are indistinguishable from those of veratrinized nerves Single, brief shocks elicit repetitive responses with an augmented negative afterpotential Summation and fatigue of the veratrinic component are similar to those seen in veratrinization Accommodation is significantly affected, and the anodal closing

threshold is decreased until it may be lower than the cathodal closing threshold Calcium antagonizes the responses to tetraethyl ammonium (2, 41) Occasionally a veratrinic mechanogram may be obtained with this substance in the frog sartorius muscle (6) Tetraethyl ammonium resembles veratrum alkaloids also in causing a positive inotropic effect in the failing mammalian heart and, with higher doses, cardiac irregularities (4) On the blood pressure it has effects superficially similar to those of veratrum alkaloids but due to a different mechanism (5)

In 9-phenanthroate and some other 9-phenanthrene derivatives, Eddy (57) and Smith (239) found substances which in mammals cause a condition like human myotonia No veratrinic mechanograms could be obtained from frog muscle, but the mechanical response of rabbit muscle, stimulated directly or indirectly, was like that found after veratrine After curarization, the veratrinic response occurred on direct stimulation Summation and fatigue occur in a manner similar to that described above for veratrine Quinine opposes these veratrinic muscular effects Unlike veratrine 9-phenanthroate has no effect on the arterial pressure and heart rate of anesthetized cats and dogs (3)

Dihydronapththacridine carbonic acid and a group of related substances (32) resemble closely the 9 phenanthroate in its veratrinic effects (113) It produces a myotonic syndrome in mammals which is associated with veratrinic responses of skeletal muscles that persist on direct stimulation after curarization or denervation Summation and fatigue of the veratrinic component occur as with 9-phenanthroate or in clinical myotonia Quinine opposes the veratrinic muscular effects (3) Anodal closing threshold is rendered lower than cathodal closing threshold, and a slowly accommodating response occurs on stimulation of muscles with direct current (175) In Europe this substance was used under the name "Tetrophan" in clinical neurology when a strengthening of muscular contraction was desired (see Foerster, 73)

Gasser (83) states that "the fact that such varied chemical entities come into a common group on the basis of their imitation of the action of veratrin indicates that they are revealing a fundamental characteristic of skeletal muscle" (See also 252) In the light of the discussion of section VI, A3, p 412 we may tentatively revise this to apply also to nerve, or to the excitatory and conduction system of nerve and skeletal muscle Further study may reveal dissimilarities among the veratrinic responses which will lead to a better understanding of this fundamental characteristic[4]

## REFERENCES

(1) Aalsers T G Contribution à la connaissance de l'action de la vératrine sur les muscles de grenouille Arch néerl de physiol 17: 435 1932.
(2) Acheson G H Unpublished observations, 1944

[4] As this goes to press we learn of the paper by Coppée (Arch internat de physiol 53: 327 1943), parts of which deal with the action of veratrine on nerve and muscle Welsh and Gordon (Fed Proc 5 112 1946) have reported that DDT and pyrethrins in common with veratrine produce repetitive responses to single shocks in lobster nerve Bucher (unpublished observations) has found that in mammals 2-4-dichlorphenoxyacetic acid produces veratrinic muscular responses

(3) ACHESON, G H AND G K MOE Unpublished observations, 1944
(4) ACHESON, G H AND G K MOE Some effects of tetraethyl ammonium on the mammalian heart J Pharmacol and Exper Therap 84 189, 1945
(5) ACHESON, G H AND G K MOE The action of tetraethyl ammonium ion on the mammalian circulation J Pharmacol and Exper Therap , in press, 1946
(6) ACHESON, G H AND M A ROOT Unpublished observations, 1944.
(7) ACHESON, G H AND A ROSENBLUETH Some effects of veratrine upon circulated mammalian nerves Am J Physiol 133 736, 1941
(8) AMANN, A AND A JARISCH Auslösung des Bezold-Effektes durch Ionen. Arch f exper Path u Pharmakol 201 46, 1943
(9) ARVANITAKI, A AND A FESSARD Sur les potentials retardés de la réponse du nerf de Crabe Action de la vératrine et de la privation d'électrolytes Compt rend Soc de biol 118 419, 1935
(10) BACKMANN, E L Action de la vératrine sur l'intestin et l'uterus et sur l'importance à cet égard des ions potassium et calcium Compt rend Soc de biol 90 128, 1924
(11) BACQ, Z M Ions et corps vératriniques Arch internat de pharmacodyn et de thérap 63 59, 1939
(12) BACQ, Z M AND G L BROWN Pharmacological experiments on mammalian voluntary muscle, in relation to the theory of chemical transmission J Physiol 89 45, 1937
(13) BACQ, Z M AND M GOFFART Transmission humorale de la contraction vératrinique Arch internat de physiol 49 189, 1939
(14) BACQ, Z M AND M GOFFART L'empoisonnement par l'aconitine et la vératrine Son mécanisme et son traitement Liége med 33 317, 1940
(15) BAYLISS, L E , S L COWAN AND D SCOTT The action potentials in *Maia* nerve before and after poisoning with veratrine and yohimbine hydrochlorides J Physiol 83 439, 1934–1935
(16) BERK, L AND M WACHSTEIN Untersuchungen am Purkinjefaden. Ztschr f d ges exper Med 89 214, 1933
(17) BERNHEIM, F AND M L C BERNHEIM The action of veratrine and urethane on tissue oxidations J Pharmacol and Exper Therap 48 73, 1933
(18) BEZOLD, A VON AND L HIRT Über die physiologischen Wirkungen des essigsauren Veratrins Unters physiol Lab Würzburg 1 73, 1867
(19) BIBERFELD, J Ergebnisse der experimentellen Toxikologie, Teil II Organische Substanzen A Alkaloide Ergebn d Physiol 17 133, 1919
(20) BIEDERMANN, W Beiträge zur allgemeinen Nerven- und Muskelphysiologie Vierte Mittheilung Über die durch chemische Veränderung der Muskelsubstanz bewirkten Veränderungen der polaren Erregung durch den elektrischen Strom Sitzungsb d kaiserlichen Akad d Wissenschaften, Wien, math naturwiss Cl III, 80 367, 1879
(21) BIEDERMANN, W Beiträge zur allgemeinen Nerven- und Muskelphysiologie Fünfte Mittheilung Über die Abhängigkeit des Muskelstromes von lokalen chemischen Veränderungen der Muskelsubstanz Sitzungsb der kaiserlichen Akad d Wissenschaften, Wien, math -naturwiss Cl III, 81 74, 1880
(22) BIEDERMANN, W Elektrophysiologie Gustav Fischer, Jena, 1895
(23) BISHOP, G H Action of nerve depressants on potential J Cell and Comp Physiol 1 177, 1932
(24) BLOUNT, B K The veratrine alkaloids Part I The constitution of veratridine Part II Cevine J Chem Soc London 122, 1935
(25) BLOUNT, B K AND D CROWFOOT The veratrine alkaloids Part III The preparation of cevanthrol and the x-ray crystallographic examination of cevanthrol and cevanthridine J Chem Soc London 414, 1936
(26) BOEHM, R Über die Wirkungen des Veratrin und Protoveratrin Arch f exper Path u Pharmakol 71 269, 1913

(27) Boehm, R. Veratrin und Protoveratrin. Heffter s Handbuch der exper Pharmakologie 2: Part 1, 249 1920
(28) De Boer, S Über das Elektromyogramm der veratrinisierten Muskeln Ztschr f Biol 61: 143 1913
(29) De Boer, S The influence of veratrine poisoning on the electrogram of the frog's heart J Physiol 49 310 1914–1915
(30) Bosetti, E II Studien über das officinelle Veratrin Arch d Pharmacie 221: 81 1883
(31) Bottazzi, P Über die Wirkung des Veratrins und anderer Stoffe auf die querge streifte, atriale, und glatte Muskulatur Arch f Physiol 377 1901
(32) Braun, J von and P Wolff Cyclische Analoga des Atophans Ber d deutsch Chem Gesellschaft 555: 3675 1922
(33) Bremer, F Researches on the contracture of skeletal muscle J Physiol 76 65 1932
(34) Bremer F and J Moldaver. La contracture neuro-musculaire de Rana Temporaria et le mécanisme de la transmission neuro musculaire Compt rend Soc de biol 127 352, 1938
(35) Brown, G L. and A M Harvey Congenital myotonia in the goat Brain 62: 341 1939
(36) Carvallo J and G Weiss Action de la vératrine sur le muscle blanc et le muscle rouge du lapin Compt rend Soc de biol 50: 553, 1898
(37) Cicardo V H Acción de la Acetilcolina intraarterial sobre el gastrocnemio del sapo curarizado con diversas substancias Rev Soc argent de biol 14: 331 1938
(38) Collins, R J The clinical actions of veratrum Arch Int Med 16: 54, 1915
(39) Collins R J and P J Hanzlik Further observations on the clinical actions of veratrum J Pharmacol and Exper Therap 11 89 1918
(40) Cowan S L The effect of veratrine on the electric response of crab s nerve J Physiol 77 27P 1933
(41) Cowan S L and W G Walter The effects of tetra-ethylammonium Iodide on the electrical response and the accommodation of nerve J Physiol 91: 101 1937
(42) Craig L C and W A Jacobs The veratrine alkaloids XII Further studies on the oxidation of cevine J Biol Chem 141 253, 1941
(43) Craig, L C and W A Jacobs The veratrine alkaloids XIII The dehydrogenation of protoveratrine J Biol Chem 143: 427 1942
(44) Craig L C and W A Jacobs The veratrine alkaloids XIV The correlation of the veratrine alkaloids with the solanum alkaloids Science 97 122, 1943
(45) Craig L C and W A Jacobs The veratrine alkaloids XVII On germine Its formulation and degradation J Biol Chem 148 57, 1943
(46) Craig L C and W A Jacobs The veratrine alkaloids XX. Further correlations in the veratrine group The relationship between the veratrine bases and solanidine J Biol Chem 149 451 1943
(47) Cramer, W On the action of Veratrum Viride With some remarks on the inter relationship of the medullary centers J Pharmacol and Exper Therap 7: 63 1915
(48) Dastre A and J P Morat Mémoires de la Soc de Biol 479 1877
(49) Denny Brown D and S Nevin The phenomenon of myotonia. Brain 65: 1 1941
(50) Denny Brown D and J Pennybacker. Fibrillation and fasciculation in voluntary muscle Brain 61: 311 1938
(51) Dixon, W E. and T G Brodie Contributions to the physiology of the lungs Part I The bronchial muscles their innervation and the action of drugs upon them J Physiol 29: 97, 1903

(52) DRURY, A N AND W S LOVE The supposed lengthening of the absolute refractory period of frog's ventricular muscle by veratrine Heart 13 68, 1926
(53) DUN, F T AND T P FENG Studies on the neuromuscular junction XIX. Retrograde discharges from motor nerve endings in veratrinized muscle Chinese J Physiol 15 405, 1940
(54) DUN, F T AND T P FENG Studies on the neuromuscular junction XX The site of origin of the junctional after-discharge in muscles treated with guanidine, barium, or eserine Chinese J Physiol 15 433, 1940
(55) ECCLES, J C The nature of syaptic transmission in sympathetic ganglion J Physiol 103 27, 1944
(56) ECCLES, J C AND W J O'CONNOR Responses evoked by a nerve volley in mammalian striated muscle J Physiol 94 7P, 1938
(57) EDDY, N B Studies of phenanthrene derivatives I A comparison of phenanthrene and some 2-, 3-, and 9-*mono-substitution products* J Pharmacol and Exper Therap 48 183, 1933
(58) EICHLER, W Veratrinkontraktur und Endplattenrhythmik Ztschr f Biol 99 243, 1938
(59) EINTHOVEN, W Die Aktionsströme des Herzens (Elektrokardiogramm) Handbuch der normalen und pathologischen Physiologie 8 (2) 820, 1928 Berlin, Springer
(60) ELLIS, C H, C H THIENES AND C A G WIERSMA The influence of certain drugs on the crustacean nerve-muscle system Biol Bull 83 334, 1942
(61) ENGELMANN, T Versuche über irreziproke Reizleitung in Muskelfasern Pflüger's Arch 62 400, 1895
(62) ERB, W Die Thomsen'sche Krankheit (Myotonia congenita) Leipzig, 1886
(63) ERLANGER, J AND H S GASSER Electrical signs of nervous activity Univ of Penna Press, Philadelphia, 1937
(64) FALK, E See FREUND, M AND H P SCHWARZ (76)
(65) FENG, T P The universal antagonism between calcium and curarizing agencies Chinese J Physiol 10 513, 1936
(66) FENG, T P *The propagation of contractures of the veratrine type* Chinese J Physiol 10 535, 1936
(67) FENG, T P Further observations on the propagation of veratrine contracture Chinese J Physiol 13 239, 1938
(68) FENG, T P The production of prolonged after-discharge in nerve by veratrine Chinese J Physiol 16 207, 1941
(69) FENG, T P AND T H LI Cross-excitation between mammalian medullated nerve fibers after treatment with Veratrine Proc Soc Exper Biol and Med 45 870, 1940
(70) FENG, T P AND T H LI Studies on the neuromuscular junction XXIV The repetitive discharges of mammalian motor nerve endings after treatment with veratrine, barium and guanidine Chinese J Physiol 16 139, 1941
(71) FENN, W O The rôle of potassium in physiological processes Physiol Rev 20 377, 1940
(72) FICK, A AND R BOEHM See FICK, A Myothermische Untersuchungen aus den physiologischen Laboratorien zu Zürich und Würzburg Wiesbaden, 1899, p 75
(73) FOERSTER, O Über die therapeutische Verwendbarkeit des Tetrophans Klin Wchnschr 55, 1925
(74) FONTES, J Action de la vératrine et dualisme fonctionnel des éléments musculaires J de physiol et de path gen 23 292, 1925
(75) FREUND, M Beitrag zur Kenntnis des Cevadins Ber d deutsch chem Ges 37 1946, 1904
(76) FREUND, M AND H P SCHWARZ Beitrag zur Kenntnis des Cevadins Ber d deutsch chem Ges 32 800, 1899

(77) Freundlich, H Kapillarchemie und Physiologie Ztschr f Chemie u Industrie der Kolloide 2 65 1907–1908
(78) Frey, M von Studien über die Wirkungsweise des Veratrins auf den quergestreiften Muskel Sitzungsber d phys med Gesell Würzburg 48 1912
(79) Fried J R G Linville and R. C Elderfield Studies on lactones related to the cardiac aglycones VII Synthesis of 3, 14 bisdesoxythevetigenin and of 14-desoxythevetigenin J Organic Chem 7: 362 1942
(80) Führer H Pharmakologische Untersuchungen über das Colchicin und seine Derivate Arch f exper Path u Pharmakol 72: 228 1913
(81) Garten S Über das elektromotorische Verhalten von Nerv und Muskel nach Veratrinvergiftung Pflüger's Arch 77: 485 1899
(82) Garten S Beiträge zur Physiologie des elektrischen Organes der Zitterrochen Abhandlungen d math phys Classe der königlich sächsischen Gesellschaft d Wiss 25 253 1899
(83) Gasser H S Contractures of skeletal muscle Physiol Rev 10: 35, 1930
(84) Gasser H S Recruitment of nerve fibers Am J Physiol 121 193 1938
(85) Gasser H S and H Grundfest Action and excitability in mammalian A fibers Am J Physiol 117 113, 1936
(86) Gasser, H S, C H Richards and H Grundfest Properties of the nerve fibers of slowest conduction in the frog Am J Physiol 123 209 1938
(87) Ghirardi G E La propagazione dell'eccitamento causato da veratrina o nicotina nel sartorio di bufo Arch di sc biol 13: 185 1929
(88) Gilbert-Queralto J and L Pescador. Über die Wirkung von Veratrin auf das Säugetierherz Arch f exper Path u Pharmakol 183 30, 1936
(89) Gilson, A S Jr and E Irvine-Jones The effects of cold and of veratrine upon the action potential of ventricular muscle Am J Physiol 92 165, 1930
(90) Goldenberg M and C J Rothberger Über die Wirkung von Veratrin auf den Purkinjefaden Pflüger s Arch 238 137, 1936
(91) Goldenberg M and C J Rothberger Weitere Untersuchungen über das Nach potential am Purkinjefaden (Arsen Chlorbarium Dioxan Aconitin) Pflüger s Arch 239 156, 1937
(92) Goldstein A and S Ellis Unpublished observations, 1943
(93) Graham, H T Modification of nerve after potential and refractory period by changes of ionic environment new cases of physiological antagonism between univalent and bivalent cations Am. J Physiol 104: 216 1933
(94) Graham, H T Supernormality a modification of the recovery process in nerve Am J Physiol 110: 225 1934
(95) Graham H T Agents modifying the effect of subthreshold induction shocks Am J Physiol 113: 52, 1935
(96) Graham H T The effects of polarization on nerve action potentials J Neurophysiol 5 137 1942.
(97) Graham, H T and H S Gasser. Modification of nerve response by veratrine protoveratrine, and aconitine J Pharmacol and Exper Therap 43: 163 1931
(98) Gregor A Über den Einfluss von Veratrin und Glycerin auf die Zuckungskurve funktionell verschiedener Muskeln Pflüger's Arch 101: 71 1904
(99) Grundfest H The properties of mammalian B fibers Am J Physiol 127: 252 1939
(100) Grundfest, H and H S Gasser. Properties of mammalian nerve fibers of slowest conduction Am J Physiol 123 307 1938
(101) Grundig J Zur Kenntnis der kombinierten Wirkung von Veratrin und Kurare auf den quergestreiften Froschmuskel Ztschr f Biol 87: 239 1928
(102) Guttman R Stabilization of spider crab nerve membranes by alkaline earths as manifested in resting potential measurements J Gen Physiol 23: 343, 1940
(103) Haas, H T A. Zur Pharmakologie des Germerins und seiner Spaltprodukte I Mitteilung Arch f exper Path u Pharmakol 189 397 1939

(104) HANZLIK, P J AND F DE EDS Pharmacology of veratrum californicum Proc. Soc Exper Biol and Med 24 557, 1926–1927
(105) HARTREE, W AND A V HILL The heat-production and the mechanism of the veratrine contraction J Physiol 56 294, 1922
(106) HARVEY, A M The actions of quinine on skeletal muscle J Physiol 95 45, 1939
(107) HARVEY, A M The relation between drug action and the calcium potassium ratio in striated muscle J Pharmacol and Exper Therap 68 494, 1940
(108) HEDBOM, K Über die Einwirkung verschiedener Stoffe auf das isolierte Säugethier herz Zweite Abhandlung Die Einwirkung gewisser Pflanzengifte Skandi nav Arch f Physiol 8 169, 1898
(109) HEINZ, R Beitrag zur Kenntnis des Cevadins See FREUND, M (75)
(110) HENZE, M Der chemische Demarkationsstrom in toxikologischer Beziehung Pflüger's Arch 92 451, 1902
(111) HERMAN, M The spread of activity in veratrinized muscle Arch internat de pharmacodyn et de thérap 53 105, 1936
(112) HERMANNS, L Toxikologische Untersuchungen an bioelektrischen Strömen 2 Mitteilung Die pharmakologische Spezifität des chemischen Alterationsstromes Ztschr f Biol 58 261, 1912
(113) HESSE, E Beiträge zur Pharmakologie des Tetrophan Arch f exper Path u Pharmakol 111 68, 1926
(114) HEYMANS, C Über die Physiologie und Pharmakologie des Herz-Vagus Zentrums Ergebn d Physiol 28 244, 1929 p 270
(115) HEYMANS, J F AND C HEYMANS Sur le mécanisme de la bradycardie consécutive à l'injection de digitale, strophantine et cymarine J Pharmacol and Exper Therap 29 203, 1926
(116) HOFFMANN, P Über die Aktionsströme des mit Veratrin vergifteten Muskels Ztschr f Biol 58 55, 1912
(117) HOU, C L Über die Veränderungen der Reizzeit-Spannungs-Kurve durch Narkose, Kälte und Veratrin Pflüger's Arch 226 676, 1931
(118) HUBER, K J Über die Ausscheidung subkutan einverleibter Alkaloide durch die Magenschleimhaut und die Speicheldrüsen Arch f exper Path u Pharmakol 94 327, 1922
(119) IKAWA, M , R J DICKE, T C ALLEN AND K P LINK The principal alkaloids of Sabadilla seed and their toxicity to *Musca Domestica* L J Biol Chem 159 517, 1945
(120) INONE, H Über die Wirkung der Pharmaka auf Ring- und Längsmuskulatur des ausgeschnittenen Kaninchendarmes bei ein und demselben Präparate I Mitteilung Acta scholae med univ imp in Kioto 5 175, 1922
(121) INONE, K On the special contraction of partly veratrinized muscle Jap J Med Sci , Part III, Biophysics 6 29, 1939
(122) IOTEYKO, J Études sur la contraction tonique du muscle strié et ses excitants Trav du lab de physiol , Instituts Solvay 5 229, 1902
(123) ISHIHARA, M AND E P PICK Pharmacology of Purkinje fibers J Pharmacol and Exper Therap 29 355, 1926
(124) ISHIKAWA, H The fundamental phenomena of life, 1924 Quoted from INONE, K (121)
(125) ISHIKAWA, Y Pharmakologische Untersuchungen an den überlebenden roten und weissen Kaninchenmuskeln Acta scholae med univ imp in Kioto 5 123, 1922
(126) JACOBJ, C Pharmakologische Untersuchung über das Colchicumgift Arch f exper Path u Pharmakol 27. 119, 1890
(127) JACOBS, W A Personal communication, 1943
(128) JACOBS, W A , L C CRAIG AND G I LAVIN The veratrine alkaloids XI The dehydrogenation of jervine J Biol Chem 141 51, 1941
(129) JACOBS, W A AND L C CRAIG The veratrine alkaloids XV On rubijervine and isorubijervine J Biol Chem 148 41, 1943

(130) Jacobs W A and L C Craig The veratrine alkaloids. XVI The formulation of jervine J Biol Chem 148 51, 1943
(131) Jacobs W A and L C Craig The veratrine alkaloids XIX On protoveratrine and its alkamine protoverine J Biol Chem 149: 271 1943
(132) Jacobs W A and L C Craig The veratrine alkaloids XXII On pseudojervine and veratrosine a companion glycoside in veratrum viride J Biol Chem 155 565, 1944
(133) Jacobs W A and L C Craig The veratrine alkaloids XXV The alkaloids of veratrum viride J Biol Chem 160: 555 1945
(134) Jappelli, A Influenza del magnesio sulla contrazione veratrinica del muscolo striato Boll Soc ital biol sper 2: 30 1927
(135) Jarisch A Kreislaufsteuerung durch das Herz Klin Wchnschr 1045 1941
(136) Jarisch A Die Bedeutung des Vagus für die Wirkung der Mistel und des Veratrins Arch f exper Path u Pharmakol 197: 266 1941
(137) Jarisch A and C Henze Über Blutdrucksenkung durch chemische Erregung depressorischer Nerven Arch f exper Path u Pharmakol 187 706 1937
(138) Jarisch A and H Richter Der Bezold Effekt eine vergessene Kreislaufreaktion Klin Wchnschr 185 1939
(139) Jarisch A and H Richter Die Kreislaufwirkung des Veratrins Arch f exper Path u Pharmakol 193 347 1939
(140) Jarisch A. and H Richter Die afferenten Bahnen des Veratrineffektes in den Herznerven. Arch f exper Path u Pharmakol 193 355 1939
(141) Jarisch A H Richter and H Thoma Zentrogenes Lungenödem Klin Wchnschr 1440 1939
(142) Job C Über die Auslösung des Bezoldeffektes durch Mistel Arch f exper Path u Pharmakol 202: 633 1943
(143) Judin, A Veratrinvergiftung Aktionsstrom und Wärmeproduktion der quer gestreiften Muskeln Pflüger s Arch 206: 151 1924
(144) Kennedy, F and A Wolf Experiments with quinine and prostigmine in treatment of myotonia and myasthenia Arch Neurol and Psychiat 37: 68 1937
(145) Kerry, R A and E Rost Über die Wirkungen des Natriumperchlorats Arch f exper Path u Pharmakol 29: 144 1897
(146) Kikuchi, T Über die absolute Kraft des Herzens Tohoku J Exper Med 11: 116 1928
(147) Kitano T Vergleichende Untersuchungen über das pharmakologische Verhalten verschiedener Skelettmuskeln des Frosches Arch f exper Path u Pharmakol 129 271 1928
(148) Kobert R Über die Beeinflussung der peripheren Gefässe durch pharmakologische Agentien Arch f exper Path u Pharmakol 22: 77 1887
(149) Kodera, Y and E T Brücke Zur Kenntnis und Theorie der Veratrinvergiftung Pflüger's Arch 220: 274, 1928
(150) Kolb L. C Congenital myotonia in goats Bull Johns Hopkins Hosp 63 221, 1938
(151) Kondo S Über die Gefässwirkung des Veratrins Acta scholae med univ imp in Kioto 3 19 1919
(152) Krayer, O and R. Mendez Studies on veratrum alkaloids I The action of veratrine upon the isolated mammalian heart J Pharmacol and Exper Therap 74: 350 1942
(153) Krayer O and R Mendez Unpublished experiments 1945
(154) Krayer O, G K. Moe and R Mendez Studies on veratrum alkaloids VI Protoveratrine Its comparative toxicity and its circulatory action J Pharmacol and Exper Therap 82: 167 1944
(155) Krayer O E H Wood and G Montes Studies on veratrum alkaloids IV The sites of the heart rate lowering action of veratridine J Pharmacol and Exper Therap 79: 215, 1943

(156) KRETZER, V AND J SEEMANN Über die Veratrinvergiftung des Froschherzens III Ztschr f Biol 57 419, 1912
(157) KUFFLER, S W Action of veratrine on nerve-muscle preparations J Neurophysiol 8 113, 1945
(158) KULIABKO, A Über die Erscheinung der Tonusschwankungen am isolierten Kaninchenherzen bei Veratrinvergiftung Pflüger's Arch 107 238, 1905
(159) KUNISHÔ, K Über die Wirkung des Veratrins, Pikrotoxins und Cocains auf den Kaninchenuterus in situ Folia pharmacol japon 20 85, 1935
(160) KUNISHÔ, K Über die Wirkung des Veratrin, Pikrotoxin und Cocain auf den im Körper befindlichen und auf den ausgeschnittenen Dünndarm Okayama Igakkai-Zasshi 47 629, 1935
(161) KUSNETZOW, A J Die Wirkung einiger Gifte auf die Funktion den isolierten Nebenniere Ztschr f d ges exper Med 48 679, 1926
(162) LAMM, G Untersuchungen über die Wirkung des Veratrins auf den quergestreiften Muskel I Mitteilung Ztschr f Biol 56 223, 1911
(163) LAMM, G Untersuchungen über die Wirkung des Veratrins auf den quergestreiften Muskel II Mitteilung Ztschr f Biol 58 37, 1912
(164) LAPICQUE, L AND M LAPICQUE Curarization par la vératrine, anatognismes dans la curarization Compt rend Soc de biol 72 283, 1912
(165) LAPORTA, M Azione della veratrina e dell'atropina sul tono dei muscoli scheletrici Arch di sci biol 19 249, 1933
(166) LISSAUER, H Untersuchungen über die Wirkungen der Veratrumalkaloide Arch f exper Path u Pharmakol 23 36, 1887
(167) LLOYD, D P C The origin and nature of ganglion after-potentials J Physiol 96 118, 1939
(168) LOEWI, O AND J SOLTI Über die Wirkung von Pilocarpin und Atropin auf den quergestreiften Muskel Arch f exper Path u Pharmakol 97 272, 1923
(169) LORENTE DE NÓ, R Personal communication, 1945
(170) LORENTE, DE NÓ, R AND L DAVIS, JR Electrotonus produced by direct current pulses in frog nerve Am J Physiol 133 366, 1941
(171) LUISADA, A Beitrag zur Pathogenese und Therapie des Lungenödems und des Asthma cardiale Arch f exper Path u Pharmakol 132 313, 1928
(172) MACBETH, A K AND R ROBINSON Cevadine Part I J Chem Soc London 121 1571, 1922
(173) MACHENS, A Über den Chemismus der Wirkung des Veratrin E Merck auf die quergestreifte Muskulatur Inaug Diss Giessen, 1908
(174) MACNIDER, W DEB The pharmacology of veratrum viride with certain therapeutic suggestions South Med and Surg 87 637, 1925
(175) MANN, L Therapeutische Versuche mit Tetrophan, besonders bei Multipler Sklerose Klin Wchnschr 2573, 1922
(176) MARFORI, P Influence de la vératrine cristallisée sur les contractions des muscles Arch ital de biol 15 26, 1891
(177) MARMONT, G The effect of chemical agents on the "local response" of large single crustacean axons Am J Physiol 133 376, 1941
(178) MARRAZZI, A S AND R LORENTE DE NÓ Interaction of neighboring fibers in myelinated nerve J Neurophysiol 7 83, 1944
(179) MASHIMO, S Jap J of Psychology 2 1920 Quoted from INONE, K (121)
(180) MCCARTNEY, E AND F RANSOM Sodium citrate and the action of certain drugs upon the frog's heart J Physiol 51 287, 1917
(181) MEIROWSKI, E Beiträge zur Kenntnis des galvanischen Wogens Pflüger's Arch 73 442, 1898
(182) MENDEZ, R AND G MONTES Studies on veratrum alkaloids III Qualitative and quantitative differences in the action of cevine and veratridine J Pharmacol and Exper Therap 78 238, 1943

(183) Meyerhof O Die Energieumwandlungen im Muskel IV Mitteilung Über die Milchsäurebildung in der zerschnittenen Muskulatur Pflüger s Arch 188 114, 1921
(184) Milheiro E Action du magnésium et de la vératrine sur le muscle strié de la grenouille Compt rend Soc de biol 96 889 1927
(185) Milheiro, E Les analogies d action du magnésium et du calcium dans la contracture vératrinique Compt rend Soc de biol 96 891 1927
(186) Milheiro E Action du strontium sur la contracture vératrinique Compt rend Soc de biol 98 548 1927
(187) Miyake Y Contribution to the study of veratrine contraction with special reference to the action of quinine Arb a d med Fakultät Okayama 6: 230 1939
(188) Miyake Y Influence of veratrine on the muscle contracting action of potassium Arb a. d med Fakultät Okayama 6 583 1941
(189) Moe G K. Unpublished observations 1945
(190) Moe G K. D L Bassett and O Krayer Studies on veratrum alkaloids V The effect of veratridine and cevine upon the circulation in anesthetized dogs, with particular reference to femoral arterial flow J Pharmacol and Exper Therap 80: 272 1944
(191) Moe, G K. and O Krayer Studies on veratrum alkaloids II The action of veratridine and cevine upon the isolated mammalian heart J Pharmacol and Exper Therap 77 220 1943
(192) Mostinsky B Die Formgesetze der Veratrinkurve des Froschmuskels Arch f exper Path u Pharmakol 57 310 1904
(193) Nachmansohn D On the effect of drugs on cholinesterase J Biol Chem 159 239 1945
(194) Paist W D E R Blout, F C Uhle and R C Elderfield Studies on lactones related to the cardiac aglycones III The properties of β-substituted Δα β butenolides and a suggested revision of the structure of the side chain of the digitalis-strophanthus aglycones J Organic Chem 6: 273 1941
(195) Pilcher, J D The bio assay of veratrum viride Am J Physiol 44 1 1917
(196) Pilcher J D and T Sollmann Studies on the vasomotor centre XVIII The effects of veratrum viride and cevadine (veratrine) J Pharmacol and Exper Therap 7 295 1915
(197) Poethke W Die Alkaloide von Veratrum album I Mitteilung Darstellung der Alkaloide und ihre Verteilung in Rhizomen Wurzeln und Blattbasen —Germerin ein neues Alkaloid von Veratrum album Arch d Pharmazie 275 357 1937
(198) Poethke W Die Alkaloide von Veratrum album II. Mitteilung Die einzelnen Alkaloide und ihre Beziehungen zueinander Protoveratridin Germerin Protoveratrin. Arch d Pharmazie 275: 571, 1937
(199) Poethke W Die Alkaloide von Veratrum album III Mitteilung Jervin Pseudojervin, Rubijervin Einteilung der Alkaloide Arch d Pharmazie 276: 170, 1038
(200) Poethke W Die amorphen Alkaloide von Veratrum album Pharm Presse 42–43 110 1937–39
(201) Prelog V and S Seifliegel Über Steroide und Sexualhormone 79 Mitteilung Über das 2 Äthyl methyl Pyridin ein Dehydrierungsprodukt des Solanidins Helvetica Chimica Acta 25: 1306 1942
(202) Prévost, J L Recherches expérimentales relatives à l'action de la vératrine Compt rend Soc de biol 18 133 1866
(203) Querido A Action de la vératrine sur le tissu musculaire strié Arch néerl de physiol 12: 28 1928
(204) Ranson, F Calcium and the action of certain drugs upon the frog s heart J Physiol 51 176 1917

(205) RAVIN, A Myotonia Medicine 18 443, 1939
(206) RICHARDS, C H AND H S GASSER After-potentials and recovery curve of C fibers Am J Physiol 113 108, 1935
(207) RICHTER, H Über die Wirkung von Oxydantien (Kaliumpermanganat, Benzoesäure peroxyd, Chinon), Veratrin und Mistel auf die Dynamik des Froschherzens Arch f exper Path u Pharmakol 194 362, 1940
(208) RICHTER, H AND A AMANN Ist die Lunge chemosensibel? Arch f exper Path u Pharmakol 196 275, 1940
(209) RICHTER, H AND H THOMA Zentrale Wirkungen des Veratrins Arch f exper Path u Pharmakol 193 622, 1939
(210) RIESSER, O Vergleichende Muskelphysiologie Ergebn d Physiol 38 133, 1936
(211) RIESSER, O AND S M NEUSCHLOSZ Physiologische und kolloidchemische Untersuchungen über den Mechanismus der durch Gifte bewirkten Kontraktur quergestreifter Muskeln II Über die durch Nikotin und Kaliumsalze ausgelöste Erregungskontraktur des Froschmuskels und über die receptive Substanz Langleys Arch f exper Path u Pharmakol 92 254, 1922
(212) RIESSER, O AND F RICHTER Weitere Beiträge zur Kenntnis der Erregungskontraktur des Froschmuskels Pflüger's Arch 207 287, 1925
(213) ROCHELMEYER, H Über Sterinalkaloide Arch d Pharmazie 275 336, 1937
(214) ROSENBLUETH, A The interaction of myelinated fibers in mammalian nerve trunks Am J Physiol 140 656, 1944
(215) ROSENBLUETH, A Recruitment of mammalian nerve fibers Am J Physiol 141 196, 1944
(216) ROSENBLUETH, A , W DAUGHADAY AND D D BOND The electrogram of the ventricle of the turtle's heart Am J Physiol 139 464, 1943
(217) ROSENBLUETH, A AND E C DEL POZO Accommodation in mammalian motor nerves Am J Physiol 136 629, 1942
(218) ROSENBLUETH, A AND E C DEL POZO The effects of veratrine upon the superior cervical ganglion Am J Physiol 136 699, 1942
(219) ROSENBLUETH, A , J H WILLS AND H HOAGLAND The slow components of the electrogram of striated muscle Am J Physiol 133 724, 1941
(220) ROSENTHAL, F , H LICHT AND F LAUTERBACH Der Mechanismus der Kühl und Krampfgifte (Ein Beitrag zum nervösen Mechanismus der Untertemperatur) Arch f exper Path u Pharmakol 106 233, 1925
(221) ROTHBERGER, C J AND A SACHS Rhythmicity and automatism in the mammalian left auricle Quart J Exper Physiol 29 69, 1939
(222) ROTHBERGER, C J AND H WINTERBERG Über scheinbare Vaguslähmung (bei Muskarin, Physostigmin, und anderen Giften, sowie bei intrakardialer Druck steigerung) Pflüger's Arch 132 233, 1910
(223) ROTHEN, A AND L C CRAIG The veratrine alkaloids XVIII Surface film studies J Am Chem Soc 65 1102, 1943
(224) SAITO, K Alkaloids of white hellebore IV Veratramine, a new alkaloid of white hellebore (*Veratrum grandiflorum* Loes fil ) Bull Chem Soc Japan 15 22, 1940
(225) SAITO, K , H SUGINOME AND M TAKAOKA On the alkaloids of white hellebore I Isolation of constituent alkaloids Bull Chem Soc Japan 9 15, 1934
(226) SAITO, K , H SUGINOME AND M TAKAOKA On the alkaloids of white hellebore II Isolation of alkaloids from the so-called resinous matters III Constitution of jervine Bull Chem Soc Japan 11 168, 1936
(227) SALZBERGER, G Über die Alkaloide der weissen Nieswurz (*Veratrum album*) Arch d Pharmazie 228 462, 1890
(228) SANTESSON, C G Einiges über die Wirkung des Glycerins und des Veratrins auf die quergestreifte Muskelsubstanz (Frosch) Skandinav Arch f Physiol 14 1, 1902

(229) SCHMITT F O AND H S GASSER The relation between the after potential and oxidative processes in medullated nerve Am J Physiol 104: 320 1933
(230) SCHÜLLER, J Warum verhindern die Lokalanästhetika die Coffeinstarre des Muskels? Arch f exper Path u Pharmakol 105 22 1925
(231) SCHÜLLER J AND F ATHERR Über den Antagonismus der Lokalanästhetika gegenüber dem Veratrineffekt am Muskel Arch f exper Path u Pharmakol 91 125 1921
(232) SCHÜTZ E. Über die Einwirkung von Arzneistoffen auf die Magenbewegungen. Arch f exper Path u Pharmakol 21 341 1886
(233) SEEMANN J AND C VICTOROFF Elektrokardiogrammstudien am veratrinvergifteten Froschherzen I Ztschr f Biol 56 91 1912
(234) SEIFERLE E J I B JOHNS AND C H RICHARDSON The alkaloids of American hellebore and their toxicity to the American cockroach. J Econ Entomol 35: 35 1942
(235) SERENI, E Sul meccanismo d'azione della veratrina Boll Soc ital biol sper 4: 1211 1929
(236) SIMON E Das Jervin eine neue Pflanzenbase Ann d Phys u Chem , Leipzig 41: 569 1837
(237) SINGH I The effect of the interaction of ions drugs and electrical stimulation as indicated by the contraction of the anterior retractor of the byssus of Mytilus Edulis J Physiol 92: 62 1938
(238) SKOGLUND C R. The response to linearly increasing currents in mammalian motor and sensory nerves Acta physiol Skand 4 supplement XII 1942
(239) SMITH, R. G Studies of phenanthrene derivatives IV A veratrine like action on skeletal muscle of certain 9-substitution products of phenanthrene J Pharmacol and Exper Therap 54: 87, 1935
(240) SOAJE ECHAGÜE E Efecto de la veratrina sobre la corriente de acción del corazón Rev Soc argent de biol 15: 373 1939
(241) SOAJE ECHAGÜE E Effets de la vératrine sur le courant d action du coeur Compt rend Soc de biol 133: 319 1940
(242) STOSHAROV, B I The effect of veratrine upon heat formation during the quiescent stage of striated muscle Arch sci biol (U.S.S R ) 44 103 1936
(243) STRAUB, W Quantitative Untersuchung des Eindringens von Alkaloiden in lebende Zellen Pflüger's Arch 98 233 1903
(244) STRAUB W Quantitative Untersuchungen über das Eindringen von Alkaloiden in lebende Zellen. Ein Beitrag zur Kenntnis des Mechanismus der Alkaloidwirkung Arch di Fisiologia 1 55, 1904
(245) SZENT-GYÖRGYI A , Z M. BACQ AND M GOFFART A humoral transmission of muscular contraction in the presence of veratrine Nature London 143: 522, 1939
(246) TANAKA K. Untersuchungen über die absolute Kraft des Herzens Hukuoka Acta Med 33: 52 1940
(247) THERMAN P O The neurophysiology of the retina Acta Soc Scient Fennicae Nova Series B 2: Nr 1 , 1938
(248) To, S Über den Einfluss verschiedener Pharmaka auf die Muskelwirkung des Veratrins Acta scholae med univ imp in Kioto 4: 31 1921
(249) TRAXLER, J Über die Ausscheidung der Alkaloide der Wurzel von Veratrum album durch Ziegenmilch Ztschr f Fleisch u Milchhyg 32: 18 1921–1922
(250) TSCHERNEWA O AND O RIESSER Über die Muskelwirkung des Kampfers nach Versuchen am isolierten Froschgastrocnemius Arch f exper Path u Pharmakol 99: 346, 1923
(251) UHLE, F C AND W A JACOBS The veratrine alkaloids. XXIV The octahydropyrrocoline ring system of the tertiary bases Conversion of sarsasapogenin to a solanidine derivative J Biol Chem. 160 243 1945

(252) VERZÁR, F AND M FELTER Untersuchungen zur Theorie der sogenannten Veratrinkontraktion Die Wirkung von Aldehyden auf die Kontraktion des quergestreiften Muskels Pflüger's Arch 158 421, 1914

(253) WACHSTEIN, M Untersuchungen am Purkinjefaden Ztschr f d ges exper Med 79 653, 1931

(254) WACHSTEIN, M Untersuchungen am Purkinjefaden II Mitteilung Experimentelle Störungen der Reizbildung und Kontraktilität Ztschr f d ges exper Med 83 491, 1932

(255) WASSILIEV, L L AND M R MOGENDOWITSCH Erregungssummation in einer alterierten Nervenstrecke Pflüger's Arch 225 389, 1930

(256) WATTS EDEN, T Untersuchungen über die Wirkungen der Veratrumalkaloide II Über die Wirkungen des Protoveratrins Arch f exper Path u Pharmakol 29 440, 1892

(257) WIBLE, C L The locus of the action of veratrin J Gen Physiol 6 615, 1924

(258) WIBLE, C L The locus of the action of veratrin in the sciatic nerve of the frog Proc Soc Exper Biol and Med 22 336, 1924

(259) WILLSON, J R AND R G SMITH The peripheral circulatory action of veratrum viride J Pharmacol and Exper Therap 79 208, 1943

(260) WOOD, H C, JR Pharmacology of veratrum J A M A 47 2061, 1906

(261) WOOD, H C, JR Is veratrum a circulatory depressant? Univ Penn Med Bull 21 27, 1908

(262) WRIGHT, C R A The alkaloids of the veratrums Part III The alkaloids of veratrum viride J Chem Soc London 35 421, 1879

(263) WRIGHT, C R A AND A P LUFF The alkaloids of the veratrums Part I The alkaloids of *Veratrum Sabadilla* (Asagroea officinalis) J Chem Soc London 33 338, 1878

(264) WRIGHT, C R A AND A P LUFF The alkaloids of the veratrums Part II The alkaloids of veratrum album J Chem Soc London 35 405, 1879

(265) WYSS, O A M Étude de la secousse vératrinique avec l'excitation selective par le temps Thesis, Paris, 1931 Quoted from HERMAN, M (111)

(266) YAMADA, S Experimentelle Untersuchungen über die Herzlähmung II Die Herzlähmung durch toxikologische Ursachen Mitt d med Fak Tokyo 26 261, 1921

(267) YOSHIDO Mitt a d med Akad zu Kioto 20 1923 Quoted from INONE, K (121)

# ARTHROPOD NERVOUS SYSTEMS A REVIEW OF THEIR STRUCTURE AND FUNCTION[1]

JOHN H WELSH AND WILLIAM SCHALLEK

*Harvard University*

The immediate purpose of this review is to bring together such literature on arthropod nervous systems, and their physiology, as might facilitate studies on the mode of action of insecticides classed as neurotoxins Of the several classes of arthropods far more is known concerning the neurophysiology of the Crustacea than of any other class This is due largely to their more favorable size and their general distribution Since such insecticides as the pyrethrins (Gaudin, 1937) and DDT (2,2 bis(p-chlorophenyl) 1,1,1 trichlorethane) (Welsh, unpublished) are more toxic to certain Crustacea than to insects, and since each substance appears to act in a similar manner in the two groups, it would seem quite justifiable to follow any suggestions which the studies on Crustacea offer in investigating the mode of action of these and other insecticides

In the development of animal poisons it is desirable to have a material with a highly selective action on the group to be dealt with. There are some striking differences between the nervous systems of arthropods and vertebrates which should be kept in mind Some of these are pointed out in the present review

Aside from any practical use which might be made of this review it stands as the only recent attempt to bring together the literature in this field

I ANATOMICAL. 1 *Gross structure* Typically the arthropod nervous system consists of *two* longitudinal cords ventral to the digestive tract with a dorsal brain A pair of ganglia occur for each somite but where the somites are fused there is often a fusion of these ganglia, and where appendages are lacking the ganglia may be absent Thus, in primitive Crustacea such as the "brine shrimp", *Artemia*, the nervous system has a ladder like form, while in higher Crustacea and insects there is lateral fusion of the parallel cords and ganglia and in crabs and certain higher insects much lengthwise shortening of the cord and fusion and loss of ganglia.

The brain is composed of the ganglia of the somites which are preoral, of paired ganglia for certain preoral, presegmental sense organs (eyes, frontal organs), and sometimes of a median anterior element, the *archicerebrum* The principal parts of the brain are the *protocerebrum, deutocerebrum* and *tritocerebrum* The identity of some of these ganglia may be lost in late stages of development

2. *General arrangement of neurons* Sensory neurons are numerous in arthropods and differ from vertebrates in having their cell bodies peripherally located They are generally bipolar The axons of sensory neurons enter the ventral ganglia through the *ventral* portion or "root" of a lateral nerve Numer

[1] This review was prepared as a part of a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the President and Fellows of Harvard College

ous association neurons are found in the ganglia and most cell bodies belong to these, as the cell bodies of sensory neurons are peripheral and those of motor neurons few in number Motor neurons are nearly all unipolar, their cell bodies are found in the peripheral portion of the ventral ganglia and the main stalk from the cell divides into a collateral and an axon filament The *typically few* motor fibers leave a ganglion by the *dorsal* "root"

3 *Peculiar features of neuromotor system* Striking differences from other animals are found in the arthropod neuro-muscular arrangements In the first place an entire muscle may be innervated by branches from only *two* motor axons which may branch and rebranch to supply many endings on *each* muscle fiber This was first shown by Biedermann (1887) in Crustacea and later extended by Mangold (1905) to a variety of arthropods Biedermann suggested that of the two nerve fibers one was excitatory and the other inhibitory This was confirmed by Hoffmann (1914) for Crustacea Certain muscles of crustaceans may be innervated by three, four, or five motor fibers (van Harreveld, 1939c) When more than two motor fibers innervate a muscle all but one are excitatory and the stimulation of each separately produces a characteristic contraction and action current (e g , van Harreveld and Wiersma, 1939)

Much less is known about the motor innervation of insects than is known about the same in Crustacea Mangold (1905) using methylene blue staining described a double innervation of the thoracic and leg muscles of *Dectcus* and of the wing muscles of *Dytiscus* A recent study of the motor mechanism of the leg of *Periplaneta* by Pringle (1939) describes a double innervation of the extensor tibiae, one fiber producing a tonic contraction and the other a twitch He found no evidence for the existence of inhibitory fibers but suggests that such may possibly exist in some insect muscle It is obvious that further investigation of the histology and physiology of motor innervation of insect muscle is desirable

4 *Nature of motor nerve endings* By means of silver impregnation van Harreveld (1939a) studied the nerve endings on muscle fibers of the abductor of the dactylopodite of the crayfish This muscle has a double innervation (one fiber excitatory, the other inhibitory) It was found that these fibers branched and rebranched simultaneously to end in a "tangle" of nerve fibers enveloping each muscle fiber A single muscle fiber, 3 5 mm long, was estimated to have at least forty nerve endings This is a situation very different from that in the vertebrates where much longer fibers usually have one and, at the most, but two or three nerve endings Evidence is presented by van Harreveld (1939a) indicating that at least part, and probably all, of the muscle fibers of a triply innervated muscle receive branches from the fibers for the fast and for the slow contraction Further evidence for this, based on physiological experiments, will be presented later

Mutiple nerve endings on a single insect muscle fiber have been described by a number of authors—most recently perhaps by Marcu (1929) who found in muscle fibers (1 mm long) of the house fly as many as twenty nerve endings

Van Harreveld (1939a) concludes that "the multiple innervation of the muscle fiber makes it anatomically possible that the impulse is not conducted

along the muscle fiber as is the case in vertebrate muscle, but that in the crayfish (and probably the insect) muscle the impulse is distributed by nerve conduction to a great number of points on the muscle fiber The action current of the crayfish muscle would then not be the sign of a conducted process in the muscle fiber, but of a local excitatory process "

Wigglesworth (1939) says, regarding nerve endings in insect muscle, "when the nerves reach the ordinary muscles they break up into smaller branches which penetrate between the fibers and have their final terminations anywhere along them The nerve often ends in a conical projection, the 'end plate' or Doyère's cone, the nerve fibre penetrates the sarcolemma and breaks up into fine branches with varicose thickenings of all sizes and shapes These 'end plates' often contain nuclei belonging to the nerve sheath or neurilemma, and strands of cytoplasm may radiate from them into the substance of the muscle fibre. In the fibrillar indirect muscles of the wings the nerve endings are difficult to find, probably they take the form of delicate branches which end all over the fibre surface. Filiform endings of this kind may occur also in any of the types of muscle."

5 *Innervation of the heart* Brief mention may be made concerning the innervation of the arthropod heart since this organ is frequently studied in connection with drug and ion effects.

Among the Crustacea the situation in the Decapoda is best known. All marine decapods, thus far investigated, have an intrinsic cardiac ganglion composed of nine cells (five large and four small) (Alexandrowicz, 1932, Welsh, 1939) The crayfish has as many as sixteen cells composing its cardiac ganglion (Alexandrowicz, 1929) Extrinsic excitatory and inhibitory nerves from ventral ganglia (CNS) end on, and regulate the activity of, the intrinsic neurons (Alexandrowicz, 1932, Wiersma and Novitski, 1942, Smith, unpublished thesis)[1]

Among the few lower Crustacea which have been studied, only in Daphnia are ganglionic pacemaker cells apparently lacking in the heart (Prosser, 1942, Baylor, 1942)

Since the early classical studies of Carlson the *Lamulus* heart has been accepted as a typical neurogenic heart with intrinsic ganglion and extrinsic regulatory nerves This is the only arachnoid concerning which information on motor regulation of the heart is at hand

Concerning the presence of intrinsic ganglionic cells in insect hearts one may refer to Wigglesworth (1939) or to Yeager and Gahan (1937) for all that seems to be known Alexandrowicz (1926) reports the presence of ganglionic heart cells in a cockroach but most other workers have failed to obtain convincing histological evidence of the presence of such cells in other species. Yeager and Gahan (1937) suggest that the different effects of nicotine on isolated hearts of *Periplaneta* and larval *Prodenia* may be due to the presence of ganglionic cells in the former and their absence in the latter In any study involving the cardiac mechanism of insects the question of the occurrence of pacemaker neurons should be given consideration

[1] Smith R. I 1942 Studies on the cardio-regulatory nerves of decapod crustaceans Thesis. Harvard University

II THE NERVE CELL AND ITS SHEATH Although the arthropod neuron was formerly believed to differ considerably from that of the vertebrates, recent work has indicated that the difference is one of degree rather than of kind The principal investigations have been those of Schmitt and Bear, in which the polarizing microscope was used to show that the crustacean nerve, like that of the vertebrates, is surrounded by a lipo-protein sheath These and other studies are reviewed in the following section Cytoplasmic structures in neurons of Orthoptera are discussed by Beams and King (1932), while those of Crustacea are treated by Lacroix (1935) These papers contain references to earlier work on this subject Among the characteristics distinguishing the arthropod neuron from the vertebrate are the following

1 Instead of converging to form an axon hillock near the periphery of the cell, the neurofibrillae form an intracellular axon deep within the cell body

2 The Golgi apparatus does not form a reticulum, but is in discrete particles

3 A supporting network, known as the trophospongium, stretches inward from the border of the cell

Although the invertebrates were generally considered to have only unmyelinated nerves, Retzius (1888) found myelinated fibers in the crustacean *Palaemon squilla* Further work showed that as in the vertebrates, the myelin is broken by nodes between the sheath cells Unlike the vertebrates, the nucleated sheath cells are not outside the myelin, but lie between it and the axon (Retzius, 1890) In the leg nerves of *Maia*, on the other hand, Young (1936) found that the axons "are enclosed in continuous sheaths, formed of sheets of a collagen-like substance, interspersed with nuclei, containing some fat, and uninterrupted by any breaks comparable with the nodes of Ranvier "

With the aid of the polarizing microscope, Schmitt and Bear (1937) found that even the so-called "unmyelinated" fibers of vertebrates are myelinated In small fibers of the frog sciatic, the nerve sheath is mostly protein In fibers of progressively greater diameter, the amount of lipid in the sheath becomes progressively greater Hence there is a continuous gradation between the small "unmyelinated" and the large myelinated fibers The lipid micelles are oriented radially, the protein tangentially, to the cell axis

Bear and Schmitt (1937) showed that the thin sheath of crustacean axons has the same properties as the myelin sheath of vertebrates By immersing the nerve in glycerine, which has the same refractive index as protein, the protein birefringence was reduced, revealing the lipid orientation Similarly, by treatment with lipid solvents such as alcohol or ether, the lipids were removed revealing the protein orientation These methods showed that the optical properties of the sheath are caused by

1, a form birefringence resulting from the shape and orientation of the protein micelles,

2, an intrinsic birefringence of the lipid micelles

Further studies indicated that in addition to their form birefrigence, there is an intrinsic birefrigence of the protein micelles (Chinn and Schmitt, 1937) The nerve cell bodies of crustaceans are enclosed in a thin sheath continuous with

and similar in its optical properties to the axon sheath Oriented protein is also found in the cytoplasm and nuclear membrane (Chinn, 1938)

Taylor (1941) found that as in the vertebrates, the lipids of shrimp nerve sheaths are oriented radially, the proteins tangentially Likewise, as in the vertebrates, the degree of myelination increases with the size of the fiber However, a shrimp nerve sheath has much less myelin than that of a frog or cat nerve of the same diameter Small fibers of both crustaceans and vertebrates have a relatively thicker sheath than do large fibers, although a vertebrate fiber of the same diameter as a crustacean fiber has a thicker sheath. The range of values for the ventral cord fibers of the shrimp is as follows

| FIBER DIAMETER | AXON DIAMETER | AXON DIAMETER / FIBER DIAMETER | BIREFRINGENCE |
|---|---|---|---|
| $\mu$ | $\mu$ | | |
| 8 3 | 5 5 | 0 66 | −0 00002 |
| 60 0 | 51 1 | 0 85 | +0 0010 |

A different situation exists in the leg nerves of *Maia*, where Young (1936) found that large fibers have relatively thicker sheaths than small ones.

Richards (1943) showed that insect nerve cells are surrounded by a lipid sheath, probably composed of phospholipids and cholesterol Using xylol marked with a sudan stain he showed that oil-soluble insecticides, entering through the tracheae, penetrate the nerve cell through this sheath Studies on the birefringence of the insect nerve sheath (Richards, 1944) showed that its properties are similar to those of crustacean sheaths The principle differences are that both protein and lipid micelles are oriented radially, and that the birefringence of the protein in insects is less than that in crustaceans Furthermore, the insect nerve cord is surrounded by a permeable, elastic neural lamella, composed of a protein differing from that of the nerve sheath Comparative studies indicated that lipo-protein nerve sheaths are found in all the major groups of the annelid arthropod complex

III BIOCHEMISTRY Perhaps the most striking indication in the biochemistry of crustacean nerve is that its metabolism is less efficient than that of vertebrate nerve The carbohydrate reserves of crustacean nerve are much greater, as is its respiration and heat production The creatine of vertebrate nerve is replaced by arginine in crustacean nerve Hence crustacean nerve is much more fatigable than vertebrate nerve

Other differences are that crustacean nerve contains large amounts of unidentified anions of unknown function Acetylcholine and cholinesterase may be much more concentrated in arthropod central nervous systems than in those of vertebrates. The following section considers these and other facts in greater detail

1 *Ionic composition* When nerves are soaked in isotonic sugar solution, the Cl and most of the Na readily escapes, while the K is retained (Fenn, Cobb, Hegnauer and Marsh, 1934) This and other evidence [illegible]

that Na and Cl are concentrated outside the fibers and K inside In frog nerve the "K space" is 36 per cent of the weight of the nerve, while in crab nerve it is 75 per cent This is probably correlated with the large diameter and thin sheath of the crab axons

Some analyses made by the above authors follow The values for crab blood are from Cole (1940), while those for insect blood are from data quoted by Heilbrunn (1943)

| | Na | K | Ca | Mg |
|---|---|---|---|---|
| | *mM /kgm* | *mM /kgm* | *mM /kgm* | *mM./kgm* |
| Crab blood | 460 | 12 | 12 | 22 |
| Crab nerve | 168 | 158 | 67 5 | — |
| Nerve/blood ratio | 0 35 | 13 | 5 6 | — |
| Frog plasma | 103 8 | 2 5 | 2 0 | 3 0 |
| Frog nerve | 62 0 | 48 0 | 3 6 | 8 0 |
| Nerve/blood ratio | 0 60 | 19 | 1 8 | 2 7 |
| Insect blood | 22 3 | 39 7 | 9 2 | 7 1 |

In keeping with the ion content of their bloods, the total ion content of crab nerve is greater than that of frog nerve Na and K are less concentrated relative to the blood in crab nerve than in frog nerve, while Ca is more concentrated The high concentration of K in the nerves of these animals is particularly noteworthy In view of the high concentration of K relative to other ions in insect blood, studies of the ionic composition of insect nerve would be of great interest

Analysis of the balance between anions and cations in lobster nerve (Schmitt, Bear and Silber, 1939) gives the following results

| | LOBSTER NERVE | SEA WATER |
|---|---|---|
| | *mEq /g* | *mEq./g* |
| Total base | 0 56 | 0 58 |
| Cl | 0 17 | 0 52 |
| Anion deficit | 0 39 | 0 03 |

Further studies indicate that this anion deficit is composed of free amino acids The concentration of these acids in lobster nerve is 60× that in the blood (Silber and Schmitt, 1940) It is suggested that these anions may play a rôle in maintaining bioelectric potentials Free amino acids are not found in medullated nerve, where their place may be taken by phospholipids Silber (1941) found that while $\frac{1}{3}$ of this anion deficit could be accounted for by amino acids, particularly alanine and aspartic acid, the other $\frac{2}{3}$ was composed of as yet unknown compounds In connection with the above work, it is interesting to note that choline acetylase, the enzyme which synthesizes acetylcholine, is stimulated by glutamic and other amino acids (Nachmansohn and John, 1945)

2 *Carbohydrates* Crab leg nerve is much richer in carbohydrate than is vertebrate medullated nerve The following data are from Holmes (1929)

| | CANCER LEG NERVE | VERTEBRATE MEDULLATED NERVE |
|---|---|---|
| | mgm./100 g | mgm./100 g. |
| Glycogen | 299–2192 | 59 |
| Free carbohydrate | 206–239 | 42 |

Glycogen may comprise 20 per cent of the total solid in crab nerve. In mosquito larvae, Wigglesworth (1942) found that glycogen is stored in the ganglia and axons of the central nervous system, occurring in the form of granules and small vacuoles This material is used up in starvation, but is reformed on feeding starch or alanine

3 *Lipids.* The lipids of the honey bee brain are remarkably similar to those of the brain of a young vertebrate prior to the appearance of visible medullation The chief difference is that the sterols are lower (Patterson, Drumm and Richards, 1945)

4 *Proteins* Lobster leg nerve contains 12 6 mgm /g total N (Schmitt, Bear and Silber, 1939) This compares with 13 0 mgm /g in the spinal cord and 20 6 mgm /g in the peripheral nerve of cattle (Abderhalden and Weil, 1912)

A protein complex with the properties of a nucleoprotein was extracted from lobster leg nerve by Bear, Schmitt and Young (1937) It is unstable in acid, precipitating as a metaprotein A histone or protamine-like group is split off in alkali A fraction containing P and characteristic of a nucleic acid can be split off the bulk of the protein The same complex was extracted from squid giant fiber axoplasm and mammalian central nervous system. Since it is apparently characteristic of neuroplasm generally, it was called *neuronin*

5 *Acetylcholine (Ach) and Cholinesterase (ChE)* Marnay and Nachmansohn (1937) found that the ChE content of lobster ventral nerve cord is 20× that of frog sciatic. This is the specific ChE which splits Ach more rapidly than other esters, and not a less specific esterase which splits other esters more rapidly than Ach (Nachmansohn and Rothenburg, 1945)

Ach was extracted from crab nervous tissue by Welsh (1939a) and from crayfish and lobster nervous tissue by Smith (1939) Smith obtained the following values

| | FIBERS | GANGLIA |
|---|---|---|
| | γ/g | γ/g |
| Cambarus fall | 4 | 14 |
| Cambarus spring | 13 | 46 |
| Homarus spring | 9 | 17 |

Schallek (1945) found that the ventral ganglia of the crab *Cancer irroratus* averaged 3 1 γ/g while the ventral nerve cord of the lobster *Homarus americanus* averaged 15 9 γ/g Corteggiani and Serfaty (1939) found Ach and ChE in the nervous systems of various insects, obtaining as much as 200 γ/g Ach in *Carausius* Comparable amounts were found in the ventral nerve cord of *Periplaneta* by Mikalonis and Brown (1941)

These values stand in great contrast to those in the vertebrate central nervous system MacIntosh (1941) found that the Ach level of the cat brain varies from 0 18 $\gamma$/g in the cerebellum to 7 0 $\gamma$/g in the basal ganglia Higher values are found in the peripheral nervous system, reaching 40 $\gamma$/g in automomic ganglia Welsh and Hyde (1944b) obtained up to 800 $\gamma$/g in the myenteric plexus of the guinea pig

Ach occurs in both the free form and in an inactive form in which it is bound to protein Although assays of arthropod nerve by Corteggiani (1938) and Corteggiani and Serfaty (1939) indicate that all the Ach in these nerves is in the free form, Schallek (1945) found that in the ventral nerve cord of *Limulus* about 40 per cent of the Ach was in the free form, of *Cambarus*, about 50 per cent, and of *Homarus*, about 60 per cent In the brain of the adult rat from $\frac{1}{3}$ to $\frac{1}{2}$ of the Ach is in the free form (Welsh and Hyde, 1944a), while in the sciatic nerve about $\frac{2}{3}$ is in the free form (Prajmovsky, unpublished)

ChE in *Melanoplus* is 8× more concentrated in the nervous system than in muscle The highest concentrations in the nervous system are found in the brain (Means, 1942) ChE appears in the *Melanoplus* embryo in small amounts before the nervous system is differentiated, and then increases rapidly as the nervous system develops Ach does not appear until later (Tahmisian, 1943)

6 *Adrenalin* Very little is known about the occurrence of adrenalin in arthropods 3, 4 dihydroxyphenylalanine, a possible precursor of adrenalin, was obtained from the larvae of the insect *Tenebrio* by Raper (1926), while a substance having the pharmacological and chemical properties of adrenalin was extracted from the same species by Wense (1938) However, as the extracts were made from whole larvae, it is not certain that these substances came from nervous tissue

7 *Metabolism* The respiration of crab nerve is 10× that of frog nerve at the same temperature (Meyerhof and Schulz, 1929) Following a 15 minute stimulation, the increased $O_2$ consumption is 20× that of frog nerve This increased respiration rises to a maximum 15 minutes after the end of stimulation, and may last for 2 hours Similar relations were found in measurements of the heat production of crab nerve (Beresina and Feng, 1933) The resting heat is 3× that of frog nerve The total heat liberated by a short stimulus is over 30× that of frog nerve While recovery heat in the frog starts at a maximum, that in the crab starts low and gradually rises to a peak Crab nerve is much more susceptible to fatigue than frog nerve This seems to be correlated with the slow recovery heat of crab nerve At low temperatures, where the recovery heat is even more prolonged, fatigability is more pronounced

Bronk (1932) showed that during a series of contractions in crab muscle, the heat production decreases, due to a slowing of the muscle This increasing economy of contraction is much greater than in vertebrate striated muscle, it enables the animal to maintain a sustained contraction despite the rapid fatigue of its nerve Furthermore, the crustacean muscle can produce either a rapid twitch or a slow contraction Keighly and Wiersma (1941) found that for similar work the slow contraction is half as expensive as the fast

Monaghan and Schmitt (1931) showed that while *Limulus* and *Homarus* muscle contains cytochrome, their leg nerve contains a hemochromagen which is not cytochrome Frog sciatic nerve has no hemochromagen but contains a carotinoid which may be carotin This pigment seems to be involved in oxidative processes.

The buffering properties of crab nerve and muscle were studied by Cowan (1933) The $CO_2$ combining capacity of crab nerve is nearly the same as summer frog nerve, while that of crab muscle is 3 per cent greater than frog muscle

Engel and Gerard (1935) found that the creatine of vertebrate nerve is replaced by arginine in crustacean nerve This implies that the crustaceans have a less efficient metabolism than the vertebrates On stimulation, phosphoarginine is decomposed, while during recovery in $O_2$ it is resynthesized at the expense of adenosinetriphosphate (ATP) Further studies on lobster muscle (Ochoa, 1938) revealed the following differences between its metabolism and that of vertebrate muscle

In vertebrates

$$\text{2 phosphopyruvate} + \text{adenylic acid} \rightarrow \text{2 pyruvate} + \text{ATP}$$
$$\text{ATP} + \text{2 creatine} \rightleftharpoons \text{adenylic acid} + \text{2 phosphocreatine}$$

In lobster

$$\text{phosphopyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}$$
$$\text{ATP} + \text{arginine} \rightleftharpoons \text{ADP} + \text{phosphoarginine}$$

IV Electrical Phenomena Although a considerable amount of investigation has been done on the electrical properties of arthropod nerve, general conclusions are hard to draw An outstanding feature of crustacean nerve is that it usually responds to a single stimulus with a series of impulses This may be due to its accommodation time being much slower than that of vertebrate nerve The conduction velocity of arthropod fibers is less than that of vertebrate fibers of the same diameter This is correlated with a much slower accommodation time than that of vetebrate nerve

Electrical studies of sense perception in arthropods indicate that there are primitive sound receptors which cannot distinguish between different frequencies, as well as more elaborate receptors permitting true hearing Similarly, there are primitive photoreceptors as well as true eyes

The central nervous system of arthropods is notable for the persistent rhythmic electrical discharge of isolated nerve cords Apparently certain cells in the cord discharge without sensory stimulation This spontaneous activity is greatly influenced by changes in the ionic composition of the surrounding fluid

A. *Peripheral Nerve* 1 *Nerve potentials* Cowan (1934) found that the injury potential of *Maia* (European spider crab) nerve is about 30 mV Bogue and Rosenberg (1936b) showed that the action potential in mixed *Maia* leg nerve is only about 10 per cent of this injury potential In thin sensory bundles the action potential is higher, ranging from 10 to 15 mV

The injury potential of crustacean nerve falls during stimulation or during

$O_2$ lack, recovery occurs only in the presence of $O_2$ (Furusawa, 1929) Hence the maintenance of the membrane potential depends upon oxidative processes

The K concentration inside *Maia* nerve is 13× that in the blood (Cowan, 1934) During stimulation, K leaks from the nerve Increasing the external K lowers both the injury and action potentials The resting potential also drops in the absence of $O_2$ but K does not leak from the nerve The depression in asphyxia is therefore not due to the loss of K.

Spider crab nerve is blocked at low temperature (Ets, 1936) Increasing the external K causes this block to occur at a higher temperature Crab nerve is more sensitive to K than frog nerve, since with a perfusion fluid containing 10× the normal K, crab nerve is blocked in 10 min , while frog nerve is blocked in 108–180 min This difference may be due to the thicker myelin sheath of frog nerve

The action of K in lowering the resting potential is opposed by Ca (Cowan, 1934) Ca by itself has no effect on the resting potential, but it does cause a reversible depression of excitability (Guttman, 1940) Mg increases the electrical response of crab nerve (Katz, 1936a)

Passage of the action current in crab nerve is followed by a slowly declining negativity (Levin, 1927) If a second impulse follows the first before this "retention" has passed away, the size of the second impulse is correspondingly reduced Levin considers that this negative after-potential is the electrical sign of fatigue Although present in frog nerve, it is much less marked because of the greater resistance to fatigue of this nerve During the passage of a series of impulses, the negative potential gradully declines and is replaced by a positive after-potential (Arvanitaki, 1936) In crab nerve which has been poisoned by yohimbine, a positive potential appears after a single nerve impulse (Bayliss, Cowan and Scott, 1935)

2 *Excitation* Sub-threshold stimuli set up non-propagated potential changes in crustacean nerve (Hodgkin, 1938) A weak stimulus produces a polarization potential which behaves as though it were due to passive accumulation of charge at the nerve membrane A stronger stimulus produces an additional potential behaving like a subliminal response of the nerve fiber These two potentials have different properties Thus the polarization potential falls at the end of the shock, while the subliminal response continues to rise The former is a passive result of the applied current, the latter an active response of the nerve

Bogue and Rosenberg (1936a) find that in thin sensory fibers of *Maia* nerve, the interval between the make of a constant current and the response is 23–74 msec at rheobasic strength The corresponding time in frog sciatic nerve is 1 7–2 0 msec

The following measurements of the chronaxie of arthropod leg nerves were made by Monnier and Dubuisson (1931)

| | FAST WAVE | SLOW WAVE |
|---|---|---|
| | *msec* | *msec* |
| *Limulus* | 1 20 | 3 75 |
| *Callinectes* | 0 70 | 2 15 |
| *Homarus* | 0 35 | 1 70 |

Chronaxies of nerve in the dog, for comparison, are 2 msec for sciatic vasomotor fibers and 0 15 msec for the facial nerve (data from Brücke, 1930)

Cutting a motor nerve of *Cancer* gives rise to a series of impulses, lasting from 15 sec. to 20 min. (Barnes, 1930b) Similarly, the nerve of *Callinectes* responds to a single stimulus with a series of impulses (Jasper and Monnier, 1933) Because of this, the excitation time of crustacean nerve is a "pseudo-chronaxie" when determined by muscular contraction For *Callinectes* it equals 0 6–0 8 msec.

Arvanitaki (1936) found that in *Carcinus* and *Maia*, this multiple response occurs only after the third or fourth of a series of stimuli Preceding and following this chain of spike potentials is a series of smaller potentials with the same periodicity as the spikes When the spike potential appears, it seems to be superimposed on these smaller oscillations

Solandt (1936) finds that this multiple response of crustacean nerve is correlated with a much slower accommodation time than that of vertebrate nerve. Values for the accommodation time (λ) of several nerves follow

| | msec. |
|---|---|
| Winter frog sciatic | 35 |
| *Maia squinado* | 1665 |
| *Cancer pagurus* | 3225 |
| *Homarus vulgaris* | 3710 |
| *Carcinus maenas* | 4160 |

Raising the Ca concentration of the perfusate decreases the accommodation time, although the effect is much less in crab nerve than in frog nerve Application of 15× the normal Ca to crab nerve abolished the multiple response, the nerve giving single impulses only (Katz, 1936a) Multiple response was obtained in frog nerve by either chilling the frog or placing the nerve in low Ca. Multiple response could then be abolished by raising the temperature or increasing the Ca concentration (Katz, 1936b)

3 *Conduction velocity* Some data on conduction velocity in arthropod leg nerves follow Stimulation of a nerve may give rise to several waves, due to different fiber groups conducting impulses at different velocities

| | 1ST | 2ND | 3RD WAVE | AUTHORITY |
|---|---|---|---|---|
| | *meters/sec* | *meters/sec.* | *meters/sec* | |
| *Lamulus* | 4 60 | 1 30 | | Monnier and Dubuisson, 1931 |
| *Callinectes* | 5 00 | 1 50 | | Monnier and Dubuisson 1931 |
| *Homarus* | 9 20 | 1 85 | | Monnier and Dubuisson 1931 |
| *Homarus* | 6 40 | | | Auger and Fessard 1934 |
| *Maia* | 4 20 | | | Auger and Fessard 1934 |
| *Maia* | 3 7–2 5 | 1 7–1 1 | 0 5–0 1 | Lullies 1933 |
| *Maia* | 2 5 | | | Bayliss, Cowan and Scott 1935 |
| *Maia* | 5 20 | 2 09 | 1 39 | Bogue and Rosenberg 1936b |

The nerves of *Leander serratus*, which are more heavily myelinated than the above nerves, have a conduction velocity of 18–23 meters/sec. (Holmes, Pumphrey and Young, 1941) Mammalian A fibers, in contrast, can conduct as fast as 100 meters/sec. (Grundfest, 1940)

Barnes (1932b) showed that the rapid closing reflex of the crustacean claw involves sensory fibers of larger diameter than those used in the slower opening reflex Conduction velocity, however, depends not only upon fiber diameter but also upon the degree of myelination (Taylor, 1942) The following table compares various fibers chosen to have the same conduction velocity Myelination is measured by positive birefringence

| FIBER | FIBER DIAMETER $d_1$ | AXON DIAMETER $d_2$ | $\frac{d_2}{d_1}$ | BIREFRINGENCE Na No | CONDUCTION VELOCITY | (Na No)$d_1$ |
|---|---|---|---|---|---|---|
| | $\mu$ | $\mu$ | | | *met /sec* | |
| Squid giant | 650 | 637 | 0 98 | − 0001 | 25 | |
| Earthworm giant | 100 | 90 | 0 90 | + 0010 | 25 | 0 10 |
| Shrimp giant | 50 | 43 | 0 87 | + 0024 | 25 | 0 12 |
| Frog sciatic | 10 | 7 5 | 0 75 | + 0105 | 25 | 0 105 |
| Cat saphenous | 8 7 | 6 6 | 0 76 | + 0140 | 25 | 0 12 |

It is evident that a small fiber can conduct as fast as a large one if it is more heavily myelinated

Monnier and Dubuisson (1931) find that animals can be classified according to the distance traversed by an impulse during the chronaxie of the nerve

| | *mm* |
|---|---|
| Lobster, both waves | 3 5 |
| Crab, both waves | 3 5 |
| *Limulus*, both waves | 5 0 |
| Shark, $\alpha$ wave, lateral line nerve | 8 0 |
| Frog, $\alpha$ wave, sciatic nerve | 12 0 |
| Dog, $\alpha$ wave, femoral nerve | 16 0 |

The increase in speed of conduction in the course of evolution is evident

B *Sense Perception* 1 *Touch and proprioception* Adrian (1930) recorded sensory discharges in the central nervous system of the caterpillar on touching the tail or head Proprioceptive impulses are set up in the nerves of various crustaceans on bending the limb (Barnes, 1930a, 1932a) Unlike vertebrate tension receptors, these end organs are in the skin of the joint, and show rapid adaptation Sensory impulses set up by these organs on moving the isolated limb cause a reflex motor response in the absence of the central nervous system. Possibly the motor nerve is stimulated by action potentials in the many fine sensory nerves which surround it

Prosser (1935a, c) recorded proprioceptive impulses in the crayfish following flexion of an appendage Tactile stimuli were recorded following movement of sensory hairs The excitation time of caudal sensory hairs is 0 5–1 5 msec.

Pringle (1938a, b, c) found that the fifth segment of the palp of *Periplaneta* is covered by hairs sensitive to touch Movement of these hairs gives rise to nerve impulses of small amplitude, their adaptation is rapid and complete A group of campaniform sensilla on each joint of the palp respond to bending of the joint or pressure on the cuticle with large, rhythmic impulses of constant height, their adaptation is slow and incomplete Other sensilla are found on the legs

2. *Hearing* The electrical response of insect nerves to stimulation of auditory receptors was studied by Pumphrey and Rawdon-Smith (1936) The tympanal organ of the locust responds to tones over a frequency range of 500–11,000 cycles/sec. Since the response is asynchronous at all frequencies the organ is unable to distinguish between different frequencies The anal cercus of the cricket and cockroach, on the other hand, responds synchronously over a wide frequency range. No lower limit of frequency could be found, and there is little response above 3000 cycles/sec In the lower range of frequency the response is almost completely synchronized with the stimulus Occasionally, however, short bursts of nerve activity will occur at twice the stimulus frequency, while the response to high frequencies may have only one half or one-quarter the frequency of the stimulus

3 *Photoreceptors* Prosser (1934b) showed that the sixth abdominal ganglion of the crayfish acts as a photoreceptor Illumination of the ganglion causes a burst of impulses which reach a maximum in about 2 sec , adaptation is slow and incomplete The activity continues for several seconds after the light is turned off The response of the eye, as recorded in the optic nerve, is quite different. A rapid burst of impulses starts 0 03 sec after turning on the light The frequency reaches a peak within 0 1 sec., and then falls off rapidly reaching a new level in about 0 5 sec A similar but smaller burst occurs when the light is turned off

Although visual physiology is outside the scope of this review, the following electrical studies in arthropods may be mentioned *Limulus* (Hartline and Graham, 1932), *Dytiscus* (Adrian, 1937), *Melanoplus* (Roeder, 1939), various insects (Crescitelli and Jahn, 1942)

C *Central Nervous System* 1 *Excitation and conduction* Although injury to peripheral nerve in the crayfish is followed by a high frequency discharge which may last several minutes, cutting a central commissure produces an asynchronous discharge lasting only a few seconds (Prosser, 1934a) Furthermore, this injury response can be obtained in an isolated claw nerve, but not in an isolated central commissure.

Conduction velocity in the ventral nerve cord of the crayfish is 3–10 meters/sec. (Prosser, 1935c) This is somewhat higher than the velocities found in arthropod peripheral nerve The time per segment is 4 0–5.5 msec of which ganglionic delay is 3.5–4 5 msec. A few fibers however, pass through each ganglion except the last without synapse

Afferent impulses set up by stimulation of caudal sensory hairs were recorded as they entered the caudal ganglion The response adapts at a steady rate during repetitive stimulation and fails to synchronize with the stimulus at frequencies above 100/sec. The non functional recovery period for most of the units is less than 5 msec., while the relative recovery period lasts for another 5 msec. Anterior to the ganglion, the afferent response fails to synchronize with repetitive stimuli faster than 10/sec The non functional recovery period is approximately 0 1 sec , while no relative recovery period could be observed Prosser suggests that these differences are due to fiber attenuation

Pumphrey and Rawdon-Smith (1937) studied the transmission of nerve

pulses through the last abdominal ganglion of the cockroach The post-ganglionic response is double, consisting of a small wave due to a few "through" fibers, followed by a larger wave due to fibers synapsing at the ganglion The "excitatory potential" built up at the synapse by afferent impulses reaches a maximum within 3–4 msec , since synaptic delay is never longer The effects of the potential persist at least 20 msec , as this is the maximal interval between stimuli at which summation will occur At low stimulus frequency the post-ganglionic response declines, although the pre-ganglionic response is unaltered. Increasing the frequency or intensity of stimulation will now restore the post-ganglionic response Apparently the post-ganglionic neurons become adapted to the original stimuli, and the "excitatory potential" must now reach a higher level to maintain the initial response

2 *Spontaneous activity* Rhythmic discharges in isolated arthropod ventral nerve cords have been noted by several authors

| | |
|---|---|
| Caterpillar | Adrian, 1930 |
| *Dytiscus* | Adrian, 1931 |
| *Cambarus* | Prosser, 1934a |
| *Periplaneta* | Roeder and Roeder 1939 |
| *Limulus* (cardiac ganglion) | Heinbecker, 1936 |

Roeder and Roeder (1939) noted an arrhythmic background of action currents of 30–60 mV in the isolated ventral nerve cord of the cockroach. Occasional rhythmic bursts of spikes would appear, with a frequency of 10–20/sec and an amplitude of 100–150 mV Other bursts of activity were apparently associated with the respiratory movements of the intact animal

A detailed study of spontaneous activity in the crayfish ventral nerve cord has been made by Prosser In the first of these studies (1934a) he found that there is a continual discharge of spikes of about 10 mV in amplitude and less than 1 msec. in duration Analysis of the records showed that the discharge is due to individual ganglion cells firing off at a frequency of 10–20/sec Although the discharge from each cell persists only a few seconds, shifting from one cell to another keeps the total frequency constant Apparently certain cells in the central nervous system can discharge without sensory stimulation

Prosser (1935b) showed that increasing the temperature increases the number of impulses in deafferented crayfish ganglia Prosser and Buehl (1939) found that this spontaneous activity is dependent on $O_2$, as it declines rapidly in $N_2$ The activity is depressed by cyanide and carbon monoxide, indicating that respiratory enzymes are involved

The effect of ions on this spontaneous discharge in the crayfish has been investigated by Roeder (1941) and Prosser (1943b) High K or low Ca initially stimulate the activity but later depress it Low K or high Ca stimulate fresh preparations but depress older ones Ca antagonizes the depressant action of high K but not its excitant action, neither action of low K is completely antagonized by low Ca Among the anions, thiocyanate has a marked stimulating effect Evidently the actions of these ions are quite complex

In the heart ganglion of *Limulus*, the discharge of neurons is increased by high

K or low Ca, and decreased by low K or high Ca (Prosser, 1943a) These relations seem to be somewhat simpler than in the crayfish nerve cord

V NEURO-MUSCULAR SYSTEMS A. *Crustacea* Some essential differences between crustacean and vertebrate nerve-muscle systems are listed by Ellis Thienes and Wiersma (1942)

1 Crustacean muscles are innervated by two to five axons, each of which innervates every fiber in the muscle Vertebrate striated muscle is innervated by a large number of axons, each of which innervates only a portion of the fibers in the muscle.

2. Each crustacean muscle fiber receives many branches from each of the axons innervating it, each vertebrate muscle fiber is usually innervated by a single motor ending

3 While vertebrate skeletal muscle fibers contract in only one way, crustacean skeletal muscle fibers can produce either a rapid twitch or a slow contraction

4. Vertebrate striated muscle responds to a single nerve impulse with a maximal twitch Crustacean muscle usually requires facilitation, that is, several nerve impulses are needed before contraction occurs

5 While inhibition in vertebrates is primarily a central phenomenon, that in crustaceans is peripheral Stimulation of a specific inhibitory neuron inhibits the response of the muscle to the other neurons.

These differences are considered in greater detail in the following sections.

1 *Multiple innervation* Lucas (1917) showed that *Astacus* muscle can respond to stimulation with two distinct types of contraction Pantin (1936b) found that the fast and slow contractions of the closer muscle of the *Carcinus* claw show marked differences in their rates of contraction and in their response to intensity and frequency of stimulation.

Van Harreveld and Wiersma (1936) showed that these two types of contraction are obtained by stimulation of different axons innervating the muscle. The same authors (1937) found that three of the muscles of the *Cambarus* claw show a triple innervation Stimulation of the thickest axon produces a fast contraction, of the next in size a slow contraction, and of the smallest, inhibition Other muscles in the claw have only a double innervation A different arrangement is found in the claw of crabs (Wiersma and Marmont, 1938), while the flexor of *Panulirus* shows a quintuple innervation (van Harreveld, 1939c, van Harreveld and Wiersma, 1939) Four of the fibers are excitatory, each producing a different type of response, while the fifth is inhibitory Because of these differences in innervation, conclusions drawn from experiments on one arthropod nerve-muscle system are not necessarily applicable to others

In vertebrate skeletal muscle, which is innervated by a large number of axons, different types of response are obtained by variation in the number of active axons or in the frequency of their discharge In crustacean muscle, which is innervated by only a few axons, a different type of response is elicited by each axon

Knowlton (1942) points out that the slow contraction is the more primitive type, being associated with smooth muscle. The rapid twitch develops with

striation, the primitive arthropod striated muscle may show both types of contraction

2 *Facilitation* Pantin (1934) found that the latent period of *Carcinus* leg muscle to repetitive stimulation applied directly to the muscle is 7–10 msec, comparable to the value for frog muscle When stimulated through the nerve, the latent period rises to 50–300 msec Allowing 4–5 msec for nerve conduction, it is evident that the bulk of this delay is at the neuro-myal junction Evidently many stimuli must reach the muscle before a contraction occurs

Further work (Pantin, 1936a) showed that a single nerve impulse produces little or no response in the muscle A succession of shocks causes a contraction, the rate and tension of which increase progressively with the frequency of stimulation Evidently each successive impulse brings more and more muscle fibers into action

In vertebrates, each skeletal muscle is composed of a multiplicity of motor units *As the intensity of the stimulus increases, more and more motor* units are activated, and the tension developed thereby increases Increasing the frequency of maximal stimuli develops a tetanus through fusion of successive contractions Because of the small numbers of axons in crustacean muscle, this mechanism does not hold The entire muscle acts as a single motor unit As long as stimulation is through one axon, the tension developed is independent of the intensity of the stimulus The response is governed entirely by the frequency and duration of stimulation Gradation is brought about through neuro-muscular facilitation

A single action potential in crustacean nerve produces a single action potential in muscle (Du Buy, 1935) The muscle action potential consists of a small spike, presumably due to the nerve impulse, followed by a longer potential which may be produced by a chemical mediator This latter potential seems to be an index of the facilitation process

Blaschko, Cattell and Kahn (1931) showed that the interjection of a single stimulus during low-frequency stimulation of crab nerve produces a rapid increase in muscle tension which is subsequently maintained by the low-frequency stimulus Pantin (1936b) suggests that this effect is due to facilitation Most of the muscle fibers are not excited by the initial low-frequency stimulus, but they are so facilitated that the single interjected impulse is enough to excite them Once the conduction path has been established, the low-frequency excitation is sufficient to maintain it

3 *Excitation* Wiersma and van Harreveld (1938a, b) showed that a single impulse in the thick (fast contraction) axon of the *Cancer* leg sets up an action current in the muscle, but does not cause a contraction If a second shock follows within 10 msec, a mechanical contraction occurs However, with repetitive stimulation contractions follow with intervals as long as 50 msec between shocks The thin (slow contraction) axon requires several stimuli before a muscle action current is set up

By varying the frequency of stimulation in *Cambarus*, a rate can be found at which stimulation of the fast axon causes maximal muscle action currents

without a mechanical response, while stimulation of the slow axon causes small muscle action currents with a definite contraction In the first case, the maximal action currents show that all the muscle fibers are being excited, yet no contraction occurs In the second case, only a few of the fibers are being excited, yet contraction occurs The action currents in these two cases must arise from different places in the same muscle fiber The excitation process is therefore a local one.

Van Harreveld (1939a, b) showed that each axon breaks into a 'feltwork" of branches which innervate the muscle fiber at a great many points Slight injury to this network prevents a response, indicating that the stimulus is not conducted over the muscle fiber

This situation is in sharp contrast to that in the vertebrates, where the action potential, arising at the motor end plate, spreads over the entire muscle fiber Local excitation permits a graded response in the crustacean muscle fiber, while the conducted excitation in vertebrates requires an all-or none response

Facilitation occurs independently for the muscle action current and for contraction (Wiersma and van Harreveld, 1938b) *During stimulation of the slow* fiber, the muscle action current at first increases through facilitation, and then declines as fatigue sets in At the same time the mechanical response is still increasing Evidently two distinct transmission processes are involved. In one, the nerve impulse sets up a muscle action current, while in the other the muscle action current produces a contraction

Evidence that the two types of contraction occur in the same muscle fiber comes from several sources Bergren and Wiersma (1938) showed that the phosphate and lactic acid production in the slow and fast contractions are the same when the mechanical effects are equal Evidently only one type of contractile substance is present.

Pantin (1934) showed that fatigue of one type of response also fatigues the other, while Wiersma and van Harreveld (1939) found that the mechanical response to stimulation of one motor nerve is facilitated by preceding stimulation of the other nerve Since this ' heterofacilitation" *does not occur for the muscle* action currents, it is evident that each nerve sets up its own muscle action currents (local response), but that these currents excite the same contractile mechanism

Finally, van Harreveld (1939a, b) showed that fibers in a triply innervated muscle receive branches from every axon Both fast and slow contractions were observed in the same fiber

The effect of ions on nerve muscle preparations of the crab was observed by Katz (1936a) Excess K causes a contracture, the tension developed in high K solutions equalling that obtained by a maximal stimulation through the nerve During this contracture the muscle is still excitable to direct stimulation. Excess Mg prevents this K contracture, and also has a curare-like action in blocking neuro-muscular transmission In the slow fiber of the *Cambarus* claw, Waterman (1941) showed that the amplitude of contraction is inversely proportional to the amount of perfused K. In the fast fiber the opposite re-

lation holds, the amplitude of contraction being directly proportional to the K concentration In both systems the magnitude of response was inversely proportional to the Mg concentration, while any change in Ca produced irregular results It is suggested that these ions act on the neuro-muscular junction

4 *Inhibition* Two types of inhibition are present in crustacean muscle (Marmont and Wiersma, 1938) If the inhibiting impulse arrives shortly before the exciting one, both the muscle action current and the contraction are reduced If the inhibiting impulse arrives at any other time, only the mechanical response is reduced The latter is called "simple", the former "supplemented", inhibition

Stimulation of the inhibitor nerve alone causes no electrical response in the muscle, but an after-effect is seen by the inhibition of the response to a succeeding excitatory impulse This persistence of the effect after the nerve impulse has ceased suggests that a neurohumor is involved Marmont and Wiersma suggest that an "inhibiting substance" may act against a "transmitting substance"

The effectiveness with which excitatory impulses can be inhibited was measured by Wiersma and Ellis (1942) Considerable variation was found between different systems Thus in the slow bender system of *Pachygrapsus* one inhibitory impulse suppresses three excitatory impulses, while in the slow closer system of *Cambarus* five inhibitory impulses are needed to counteract one excitatory impulse The fast closer system of *Cambarus* cannot be inhibited, this system also gives a mechanical response to a single stimulus, while the other inhibitable systems do not

Supplementary inhibition occurs in the stretcher muscle of the *Cambarus* leg but not in the closer, although both muscles are innervated by the same inhibitor A different situation was found in the crab by Wiersma and Helfer (1941) The opener muscle is supplied by two inhibitor nerves Stimulation of one inhibits both the electrical and mechanical response, while stimulation of the other inhibits the mechanical response alone The extent of inhibition is evidently determined by factors in both the nerve and the muscle

Knowlton and Campbell (1929) found that stimulation of one of two nerves in the lobster claw causes contraction of the closer and inhibition of the opener muscle, while stimulation of the other nerve contracts the opener and inhibits the closer Van Harreveld and Wiersma (1937) explained this relationship by analyzing the distribution of axons in the nerves of the crayfish (which presumably has the same innervation as the lobster) The thin nerve contains both the axon causing contraction of the opener and that causing inhibition of the closer, while the thick nerve contains axons causing contraction of the closer and inhibition of the opener This reciprocal innervation of antagonistic muscles differs from that of the vertebrates in that the inhibition is peripheral and not central

Pantin (1936c) showed that reciprocal responses in the limbs of *Carcinus* are caused by the differences in thresholds between the excitor and inhibitor nerves A weak stimulus excites the extensor, but has no action on the flexor A stronger stimulus inhibits the extensor and excites the flexor, while a still stronger stimulus

inhibits both the extensor and the flexor Such a relation does not occur in mammals.

A more complex situation was found in the crab by Wiersma (1941) All four distal muscles of the leg are innervated by the same inhibitor Clearly this nerve could not be used in reciprocal reflexes Wiersma suggests that it may be used in moulting, when relaxation of all muscles is required

Another explanation is suggested by Pantin (1936c) In the resting limb, normal tone is maintained by low frequency excitation A few extra impulses at high frequency will cause a contraction, which is then maintained by the low frequency discharge An inhibitory discharge will *stop* the contraction without interrupting the low frequency tone Instead of the continual discharge needed to maintain a vertebrate tetanus, only a few impulses are needed to start this contraction and a few more to stop it This mechanism enables the crustacean to maintain a sustained contraction at minimum expense.

B *Arachnida and Insecta.* Little work has been done on arthropod nerve-muscle systems aside from those of the Crustacea Rijlant (1934) investigated muscle activity in the scorpion At rest, the flexor muscles show a tonic activity marked by slow action potentials, while there is no activity in the extensors. During contraction, large rapid waves occur in the flexors, while tonic activity appears in the extensors Flexor tonus is inhibited during contraction of the extensors In contrast to the mammals, the flexors have a marked tonic activity, while extensor tonus appears only during contraction of the flexors

Pringle (1939) found that the neuro-muscular system of the extensor tibiae of the cockroach is like that of the crayfish in having "fast" and "slow" contractions produced by stimulation of different axons Furthermore, the same muscle fiber responds to both types of stimulation The fast axon has a lower threshold than the slow axon, and produces larger muscle action currents. The chief difference between the two animals is that no evidence for inhibitory fibers was found in the cockroach The slow axon differs from that of the crayfish in that a single impulse produces a contraction Further analysis of insect neuro-muscular systems would be of great value.

VI PHARMACOLOGY The pharmacology of the vertebrate nervous system has taken on a different aspect in the past two decades due largely to the discovery of two physiologically different types of neurons (1) those whose activity appears to depend on the formation and action of Ach, for which Dale proposed the term "cholinergic", (2) those characterized by the occurrence of adrenalin for which the term "adrenergic" was proposed Many drugs used empirically in the past may perhaps act by preventing the rapid destruction of Ach or adrenalin, by substitution, or by blocking the action of Ach or adrenalin at synapses or at neuro-effector junctions For detailed information one may refer to textbooks on pharmacology (e g , Gaddum, 1940, Goodman and Gilman, 1941)

In animals other than vertebrates too little is known about the exact distribution of cholinergic and adrenergic neurons, and the mode of action of Ach and adrenalin (as well as the possible occurrence of other "neurohumors"), to enable one to interpret the action of many of the standard drugs which affect

the nervous system Hence it is quite unsafe to assume that a given drug with a known action in the vertebrates will have a similar action in the invertebrates

A selected series of drugs, whose action on arthropod nervous systems has been studied, will be considered in this section Insofar as information allows, their actions on the heart, central nervous system and peripheral nerves will be reviewed It is difficult in the case of the arthropod heart to determine the exact locus of action of a given drug since the possible loci are several on cell bodies of pace-maker neurons (when present), on axons of these neurons, at their end ings, and directly on the heart muscle In the intact heart, possessing extrinsic regulatory nerves, the situation is even more complex

1 *Nicotine* This active alkaloid of tobacco is well known as an insecticide (Shepard, 1939) In spite of its wide use, however, little is known concerning its exact mode of action in insects Carlson (1906) applied nicotine separately to the cardiac ganglion and muscle of the *Limulus* heart When applied to the ganglion nicotine gave initial stimulation followed by irregularities of rhythm, depression, or paralysis, depending on the concentration used When applied directly to muscle, low concentrations had little effect, high concentrations were depressive The heart of the crab, *Cancer magister*, (Davenport, 1941) was found to be excited by nicotine in relatively low concentrations and depressed by high concentrations The action of nicotine on isolated heart preparations of larvae of the southern army worm, *Prodenia eridania*, and of the cockroach, *Periplaneta americana*, was studied by Yeager and Gahan (1937) They observed with both hearts that relatively low concentrations of nicotine caused stimulation, which was not followed by depression, while relatively high concentrations gave initial stimulation followed by complete paralysis The many differences ob served in the behavior of the two hearts to nicotine led these authors to suggest that " the marked differences in response of the roach and larval (*Prodenia*) isolated cardiac mechanisms to perfusing nicotine may be due to differences either in their possession of intrinsic ganglionic cells or in the resistance to nicotine of various of their component or muscular parts" Hamilton (1939) working with the heart of the grasshopper, *Melanoplus differentialis*, found that concentrations of nicotine ranging from 0 0001 to 1 0 per cent by volume caused an increase in amplitude with little change in rate Repeated applications of 1 0 per cent nicotine caused complete paralysis Yeager and Munson (1945) have recently compared the actions of nicotine and DDT in *Periplaneta* Isolated and semi-intact ventral nerve cords of arthropods such as *Cambarus* and *Periplaneta* continue to show electrical activity which is spoken of as "spon taneous" The frequency of discharge is a function of the rate of "firing" of individual neurons, as well as the number of neurons active at a given time Certain drugs have a marked effect on the level of activity Roeder and Roeder (1939) found that nicotine $10^{-5}$ produced an appreciable increase in the spontaneous activity of the isolated ventral nerve cord of *Periplaneta* Nicotine $10^{-4}$ produced great activity within fifteen seconds after application, followed in two minutes by complete blocking or paralysis Prosser (1940) observed similar effects of nicotine on the nerve cord of the crayfish

In the most complete recent study of the effect of drugs on the peripheral nervous system of arthropods, Ellis, Thienes and Wiersma (1942) observed the action of nicotine on the peripheral nerve-muscle preparation of the cheliped of the crayfish, *Cambarus clarkii*. When nicotine (0.01 mgm /g ) was injected into whole animals there was an initial increase in reflex excitability followed by depression, when nicotine (1 per cent) was applied to peripheral nerve muscle preparations there was no effect.

The evidence from the several studies suggests that nicotine acts on motor cell bodies and possibly synapses in the arthropod nervous system, causing excitation in low concentrations and depression or paralysis in high concentrations, and it has little or no action on the nerve fiber or at the nerve endings on muscle.

2. *Curare*. In the vertebrates curare has the inhibitory actions of nicotine *without* preliminary excitation. Its paralytic actions on neuromuscular junctions is much greater than that of nicotine. This action has been shown to be due to a blocking of acetylcholine at motor end plates. The effects of curare on arthropods are quite different from those on vertebrates but in many respects resemble the effects of nicotine on arthropods.

Carlson (1922) reports that curare, like nicotine, first stimulates and then paralyzes the arthropod heart after cutting extrinsic nerves. In the *Limulus* heart these drugs act primarily on the heart ganglion, not on the heart muscle or the intrinsic motor nerve fibers. No excitatory action of curare on the heart of the crab, *Cancer magister*, was observed by Davenport (1942) but high concentrations brought about periodic stoppage in diastole. Davenport also found that *previous treatment with curare greatly altered the response of the Cancer* heart to Ach: either the characteristic excitatory action of Ach was absent or a decrease in rate appeared after curare and Ach.

It has been known for many years that curare acts especially on the central nervous system of crustaceans. Thus Piotrowski (1893) observed that curare injected into a crayfish resulted in complete paralysis but a chela removed showed all of the phenomena of a normal preparation on stimulation of its nerve. Katz (1936a) has made similar observations on the crab, *Carcinus*. Ellis, Thienes and Wiersma (1942) found that the injection of curare (0.3 mgm /g ) into entire crayfish depressed reflex excitability while one per cent curare had no observable action on the peripheral neuro-muscular system of the crayfish. It appears then that in the Crustacea curare has the inhibitory actions of nicotine, usually without preliminary excitation, while both drugs fail to have any demonstrable action on peripheral nerve or myo-neural junctions.

3. *Muscarine*. This drug causes increases in amplitude and frequency of the heart of *Cancer* (Davenport, 1942) but has no peripheral action in the crayfish (Ellis, Thienes and Wiersma, 1942).

4. *Pilocarpine*. Bain (1929) found the isolated heart of *Maia* to be excited by pilocarpine and that this action was blocked by atropine. Roeder and Roeder (1939) likewise observed an excitatory action of pilocarpine on the spontaneous activity of the isolated nerve cord of *Periplaneta*, also that this action could completely blocked by atropine. Ellis, Thienes and Wiersma (1942) inject

pilocarpine (2 mgm /g ) into entire crayfish and obtained increased reflex activity but failed to obtain an effect on peripheral nerve With a limited range of concentrations of pilocarpine ($5 \times 10^{-4}$ to $5 \times 10^{-3}$) Waterman (unpublished)[3] obtained large amplitude increases in constantly perfused preparations of the flexor dactyl of *Maia*

5 *Acetylcholine (Ach)* Much interest has been centered on this highly active substance in the past two decades It has been shown, with reasonable certainty, to be involved in transmission at ganglionic synapses in the autonomic nervous system, at postganglionic nerve endings in the parasympathetic and at myo-neural junctions of skeletal muscle It probably plays a rôle in central transmission Thus far no direct evidence has been presented indicating a rôle of Ach in conduction along the nerve fiber

The actions of Ach in the vertebrates are commonly classed as "nicotine-like" or "muscarine-like" according to their resemblance to the action of these drugs For example, the action of Ach on the vertebrate heart resembles that of muscarine, while the action at motor end plates of skeletal muscle resembles that of nicotine

Arthropod nervous systems contain relatively large quantities of Ach (see section III) but except for its rôle in the control of heart beat the normal functions of Ach in this group are poorly understood It is important to keep in mind the evanescent nature of Ach in the presence of uninhibited cholinesterase, and the difficulty which must necessarily attend the attainment of appropriate concentrations of this drug at points where it may be expected to act Dale's group worked with great care and patience to demonstrate that Ach may, when properly administered, produce a normal muscle twitch

Hearts of vetebrates are inhibited by Ach, the great majority of arthropod hearts are excited The hearts of all decapod Crustacea that have been studied show an increase in amplitude or frequency, or both, following perfusion with Ach (MacLean and Beznak, 1933, Welsh, 1939a, b, 1942, Davenport, 1941, Davenport, Loomis and Opler, 1940, Wiersma and Novitski, 1942, Prosser, 1942) The heart beat of certain amphipods, isopods and copepods is accelerated by Ach (Prosser, 1942), that of *Daphnia* is slowed (Baylor, 1942), while the beat of the hearts of the primitive crustaceans, *Artemia* and *Eubranchipus* is unaffected (Prosser, 1942) The heart beat of *Limulus* is accelerated by Ach (Garrey 1942) Hamilton (1939) observed an immediate increase in frequency of heart beat in *Melanoplus* following the application of Ach Prosser suggests that hearts which are accelerated are neurogenic, those which are inhibited are innervated but myogenic, and those which are unaffected are non-innervated Most observers have noted that atropine abolishes the response of the arthropod heart to Ach The *Limulus* heart is an exception (Garrey, 1942)

It is difficult to demonstrate an action of Ach on the ventral nerve cord of arthropods at concentrations which are physiologically significant Bonnet (1938) observed an immediate augmentation in frequency and amplitude of

[3] Waterman, T H 1943 Crustacean neuromuscular transmission (Thesis) Harvard University

otentials from intact crayfish thoracic ganglia on application of Ach $10^{-9}$ to $10^{-7}$ This effect lasted for one minute and was followed by a depression lasting ne to eight minutes, depending on the concentration employed Concentra ons above $10^{-7}$ were found to have only a depressive action Prosser (1938) ad earlier failed to obtain effects of Ach on spontaneous electrical activity in eafferented abdominal ganglia of the crayfish and following the study by onnet carefully repeated his experiments using a wide range of concentrations f Ach and three species of crayfish (Prosser, 1940) He was unable to observe ny effect of Ach on spontaneous activity or synaptic transmission in abdominal anglia at concentrations of $10^{-9}$ to $10^{-4}$ inclusive Higher concentrations did ncrease the frequency of spontaneous discharge but failed to affect synaptic ransmission Furthermore Prosser (1940) failed to find any potentiation of the ch action with previous or simultaneous treatment with eserine Using the olated nerve cord of *Periplaneta*, Roeder and Roeder (1939) likewise did not ob- in an effect on spontaneous activity with either Ach or ethoxycholine until oncentrations of $10^{-4}$ or higher were used Previous treatment of the cord with serine $10^{-7}$, which is just too dilute to cause an increase in activity, did not in rease the response to Ach Because the cockroach nerve cord responds to low oncentrations of such drugs as pilocarpine, nicotine and eserine (Roeder and Roeder, 1939) and contains large quantities of Ach and cholinesterase (Milka ous and Brown, 1941) it is reasonable to assume that a cholinergic system is laying some part in its normal activity What this may be, however, remains o be determined

Several attempts have been made to ascertain whether or not Ach acts as a ransmitter substance between motor nerve and skeletal muscle in Crustacea. A variety of techniques has been employed and there has been consistent failure o detect any significant action of Ach on peripheral nerve, or on muscle (Bacq, 1935, Katz, 1936a, Ellis, Thienes and Wierama, 1942, Waterman, unpublished)

Injection of Ach into intact Crustacea may result in the autotomy of legs or increase the tendency to drop legs which are grasped by forceps (Welsh and Heakin, 1939) The exact mode of action was not determined. It might have been to increase the number of sensory impulses, to facilitate central transmission, to excite motor neurons or to stimulate the autotomizer muscle directly Ellis, Thienes and Wiersma (1942) observed that injected Ach produced ex citation (increase in reflex activity) in the crayfish

6 *Eserine* (*physostigmine*) This drug acts by blocking the enzyme cholin esterase and allowing applied Ach to act before being destroyed, or allowing an accumulation of Ach at points where it normally acts. The observation that the action of a nerve is increased by eserine may be taken as suggestive, but not con clusive evidence that the nerve is cholinergic

Restoration of a regular heart beat, and some acceleration with eserine alone, have been observed in decapod Crustacea by Welsh (1939b) and Davenport, Loomis and Opler (1940) Marked potentiation of the action of Ach after eserinization of the decapod heart has been observed (Welsh, 1939b, 1942, Davenport, Loomis and Opler 1940, Davenport, 1941) Comparison of the

acceleration of the crayfish heart produced by maximal stimulation of the accelerator nerve, before and during perfusion with eserine, showed that eserine enhanced the effect of nerve stimulation (Wiersma and Novitski, 1942)

Prosser (1940) found that eserine stimulated cells of ventral ganglia of crayfish to fire repetitively This action was usually followed by blocking of synaptic transmission Roeder and Roeder (1939) found that eserine $10^{-4}$ produced a great increase in spontaneous electrical activity of the isolated nerve cord of *Periplaneta*

Katz (1936) reported no sign of improved facilitation after eserinization ($10^{-6}$ to $10^{-3}$) of crab nerve muscle preparations Ellis, Thienes and Wiersma (1942), however, observed that eserine increased the tendency of crayfish nerve to fire repetitively These latter workers observed that perfusion with eserine initiated spontaneous contractions in the isolated chela of the crayfish

The anomuran crustacean, *Petrolisthes armatus*, was found to be extremely sensitive to injected eserine (Welsh and Haskin, 1939) Injection of 0 3 $\gamma$/g caused increased motor activity followed by recovery, 0 75 $\gamma$/g caused greatly increased motor activity and unco-ordinated movements followed by sluggishness, failure to autotomize and the death of some animals, while 1 5 $\gamma$/g caused extreme activity followed by tetanic convulsions and 100 per cent mortality

7 *Atropine* In the vertebrates this drug paralyzes most of the cholinergic nerves with muscarine-like action It does not prevent the liberation of Ach but apparently blocks "receptor substances" to prevent Ach from acting when liberated Atropine has, at first, a stimulating action on the central nervous system from the motor area to the medulla, which is followed by depression

Bain (1929) observed that atropine abolished the excitatory action of pilocarpine on the isolated crustacean heart That atropine prevents the excitation, produced by perfused Ach of a variety of decapod crustacean hearts has been observed (Welsh, 1939a, b, Davenport, Loomis and Opler, 1940, Davenport, 1941) Davenport (1942) found the action of nicotine on the heart of *Cancer magister* was not in any way modified by atropine, that atropine completely abolished the response to muscarine and acetyl-beta-methylcholine (mecholyl), but that the response to carbamylcholine (doryl) was not abolished by atropine

Carlson (1906) and Garrey (1942) have observed only an excitatory action of atropine on the cardiac ganglion of *Limulus* and Garrey found no blocking of the excitatory action of Ach after atropinization

Prosser (1940) found atropine ($10^{-4}$) had no apparent effect upon the synaptic response in the crayfish nerve cord Roeder and Roeder (1939) report that the electrical activity of the isolated nerve cord of *Periplaneta* is unaffected by atropine $10^{-3}$ but that previous atropinization completely prevents the excitatory action of pilocarpine These latter authors were unable to obtain conclusive evidence that the excitatory action of Ach was blocked by atropine

Atropine (1 per cent) was found to have an anesthetic action when injected into the isolated chela of the crayfish (Ellis, Thienes and Wiersma, 1942) Its action resembled that obtained with nupercaine, procaine, diothane and amphetamine Welsh and Haskin (1939) found that atropine injected into *Petro-*

*lathes* had a depressive action, and, depending on the amount injected, reduced or abolished the autotomy of appendages Crozier (1922) describes the action of atropine injected into lepidopterous larvae as producing a "reversal of inhibition" since a reversal in the behavior of the prolegs appeared after injection of atropine. Later Crozier and Pilz (1924) obtained general excitation of *Melanoplus* when atropine was injected or applied to thoracic ganglia.

8 *Adrenalin* This active substance from the suprarenal medulla and adrenergic nerves has many familiar actions in the vertebrates such as augmentation of rate and force of heart beat, inhibition of tone and movement of stomach and intestine, etc Its actions in arthropods have not been extensively studied Adrenalin is known to excite the decapod crustacean heart (MacLean and Bezmak, 1933, Bain, 1929, Welsh, 1939, Davenport, Loomis and Opler, 1940) Welsh, and Davenport, Loomis and Opler observed that the excitatory action of adrenalin persisted for some time during washing (20 min or more with isolated heart of *Panulirus*)

Prosser (1940) found adrenalin $10^{-4}$ to increase somewhat the spontaneous electrical activity of cells in the crayfish ventral nerve cord Ellis, Thienes and Wiersma (1942) found no effect of adrenalin on the peripheral system in the crayfish but on injection into whole animals there was marked depression of reflex excitability This agrees with observations made by Welsh and Haskin (1939) on the effect of adrenalin in depressing the autotomy reflex in *Petrolisthes*

9 *Yohimbine* This drug in a saturated solution (about 1 20,000) lengthens the refractory period of crustacean nerve (Bayliss, Cowan and Scott, 1935, Marmont, 1941, Ellis, Thienes and Wiersma, 1942)

10 *Veratrine* This mixture of alkaloids from the underground stems of the white hellebore, *Veratrum*, has a very marked action on peripheral crustacean nerve Bayliss, Cowan and Scott (1935) found that veratrine 1 25,000,000 when applied to isolated leg nerves of *Maia*, produced a prolonged after potential which persisted for one hour Ellis Thienes and Wiersma (1942) observed that veratrine tended to prolong the refractory period of crayfish nerve but more striking was the initiation of repetitive discharge which produced spontaneous twitching of claw muscle.

So far as is known no study has been made of the action of the veratrine alkaloids on the arthropod central nervous system

11 *Piperidinomethylbenzodioxane* (*933F*) This piperidine derivative of dioxane acts in vertebrates to antagonize adrenalin but at the same time stimulates the central nervous system by an adrenalin like action (Gaddum, 1940) It was found by Waterman (unpublished) to have a powerful excitatory action when perfused through the crustacean appendage. Responses of the perfused flexor dactyl, being stimulated by way of nerve, showed an amplitude increase of as much as 300 per cent. Waterman says, "It was characteristic of the responses to this drug that the contractions became delayed and relaxations slower This delay was often so great that the tension had only risen to ½ of its final value when the burst of stimuli to the nerve was already over Recovery from these large effects was slow and often incomplete" This action of 933F observed by Water

man is accounted for by the observation of Ellis, Thienes and Wiersma (1942) that this drug increases the tendency to repeated discharge by crayfish nerve.

12 *Pyrethrum* Of the insecticides which have not been mentioned in this section there is considerable information on the mode of action of the pyrethrins. The earlier literature is cited by Shepard (1939) Ellis, Thienes and Wiersma (1942) found the pyrethrins, in very low concentrations, when applied to nerve-muscle preparations of the crayfish were among the most effective substances in inducing repeated discharge of the nerve A single weak shock applied to the nerve after treatment produced a volley of muscle action potentials Injection of pyrethrins into an isolated claw initiated spontaneous contractions It is remarked by these authors that spontaneous contractions are especially pronounced with eserine and pyrethrum

## SUMMARY

A selection of a few of the more interesting and characteristic features of the arthropod nervous system follows The central nervous system consists of a "brain" formed by the fusion of paired ganglia, followed by two ventral ganglionic chains with various degrees of lateral and longitudinal fusion Sensory neurons have their cell bodies located peripherally Numerous association neurons are found in the ganglia along with the motor cell bodies which are relatively few in number Recent studies with the polarizing microscope have shown that arthropod nerve, like that of the vertebrates, is surrounded by a lipoprotein sheath The carbohydrate reserves of crustacean nerve are much greater than those of vertebrate nerve, as are its oxygen consumption and heat production It is also more readily fatigued than vertebrate nerve Evidently the metabolism of crustacean nerve is less efficient than that of the vertebrates Crustacean nerve usually responds to a single stimulus, unless of very short duration, with a series of impulses This may be due to its accommodation time being slower than that of vertebrate nerve Isolated arthropod ventral nerve cords show a persistent rhythmic discharge Apparently certain cells in the central nervous system discharge without sensory stimulation Each of the axons innervating a crustacean muscle produces a different type of response In a triply-innervated muscle, one axon produces a fast contraction, another a slow contraction, while the third inhibits the response to the other two Since each axon innervates all the fibers of the muscle, the entire muscle acts as a single motor unit The tension developed is independent of the intensity of the stimulus, and is governed entirely by its frequency and duration. Each muscle fiber receives many branches from each of the axons innervating it The excitation process is a local one, and is not conducted over the fiber Hence crustacean muscle shows a graded rather than an all-or-none response Although arthropod nervous systems contain large quantities of acetylcholine and cholinesterase it has been difficult, by means of pharmacological studies, to demonstrate precise rôles for acetylcholine Many drugs do not have comparable actions in arthropods and vertebrates For example, neither nicotine nor curare block transmission from nerve to skeletal muscle in arthropods but both sub-

stances have a central action A variety of unrelated substances, including veratrine, 933F, eserine and pyrethrum, when applied to peripheral crustacean nerve, produce multiple discharge to single weak stimuli

## REFERENCES


ABDERHALDEN E AND A WEIL. Vergleichende Untersuchungen über den Gehalt der verschiedenen Bestandteile des Nervensystems an Aminosauren I Hoppe Seyler's Ztschr physiol Chem. 81: 207 1912

ADRIAN, E D The activity of the nervous system in the caterpillar J Physiol 70: 34P, 1930
Potential changes in isolated nerve system of *Dytiscus* J Physiol 72: 132, 1931
Synchronised reactions in optic ganglion of *Dytiscus* J Physiol 91: 66 1937

ALEXANDROWICZ, J S The innervation of the heart of the cockroach (*Periplaneta orientalis*) J Comp Neurol 41: 291, 1926
Badania nad unerwieniem serca raka (*Potamobius astacus*) With French resumé Folia Morph 1: 87, 1929
The innervation of the heart of the Crustacea I Decapoda Quart J Micro Sci 75: 181, 1932

ARVANITAKI, A Variations lentes de potentiel, associées au fonctionnement rhythmique des nerfs non myelinisés isolés J Physiol et Pathol gen 34 1182 1936

AUGER, D AND A FESSARD Sur le poténtial d'action des fibres nerveuses les plus rapides chez quelques Crustacés marins C R Soc Biol 116 98 1934

BACQ Z M La choline-estérase chez les Invertébrés L insensibilité des Crustacés à l'acétylcholine Arch Inter Physiol 42: 47 1935

BAIN W A The action of adrenalin and of certain drugs upon the isolated crustacean heart Quart J Exper Physiol 19 297 1929

BARNES T C Sensory impulses in crustacean nerve J Physiol 63: 32P, 1930a
Peripheral tonus associated with impulse discharges in crustacean nerve J Physiol 70 24P 1930b
Responses in the isolated limbs of Crustacea and associated nervous discharges Am J Physiol 98: 321, 1932a
The significance of fiber diameter in the sensory nerves of the crustacean limb Am J Physiol 100: 481, 1932b

BAYLISS, L E S L COWAN AND D SCOTT, JR The action potentials in *Maia* nerve before and after poisoning with veratrine and yohimbine hydrochlorides J Physiol 83: 430 1935

BAYLOR, E R. Cardiac pharmacology of the cladoceran Daphnia Biol Bull 83: 165, 1942

BEAMS, H W AND R L KING Cytoplasmic structures in the ganglion cells of certain Orthoptera J Morphol 53: 59, 1932

BEAR, R. S AND R. O SCHMITT Optical properties of the axon sheaths of crustacean nerves J Cell and Comp Physiol 9: 275 1937

BEAR, R. S F O SCHMITT AND J Z YOUNG Investigations on the protein constituents of nerve axoplasm Proc Roy Soc B 123: 520 1937

BERESINA, M AND T P FENG The heat production of crustacean nerve J Physiol 77: 111, 1933

BERGREN, W R AND C A G WIERSMA Chemical changes in the adductor muscle of the cheliped of the crayfish in relation to the double motor innervation. J Gen. Physiol 22: 193, 1938

BIEDERMANN W Zur Kenntniss der Nerven und Nervenendigungen der quergestreiften Muskeln der Wirbellosen. Sitzungsber Akad wiss Wein, Abt III 16 8-39

BLASCHKO, H., MCK CATTELL AND J L KAHN On the nature of the two types of response in the neuromuscular system of the crustacean claw J Physiol 73: 25 1931

BOGUE, J Y AND H ROSENBERG Excitability of sensory fibres in *Maia* nerve J Physiol 87 67, 1936a
Electrical response of *Maia* nerve to single and repeated stimuli J Physiol. 87 158, 1936b
BONNET, V Contribution a l'étude du systeme nerveux ganglionaire des crustacés Arch. Inter Physiol 47 397, 1938
BRONK, D W The heat production and economy of maintained contractions in crustacean muscle J Cell and Comp Physiol 2 285, 1932
BRÜCKE, E T Vergleichende Physiologie des Erregungsvorganges Ergebn d Biol 6 327, 1930
CARLSON, A J On the point of action of drugs on the heart with special reference to the heart of *Limulus* Am J Physiol 17 177, 1906
A note on the action of curare, atropine and nicotine on the invertebrate heart J Gen Physiol 4 559, 1921–22
CHINN, P Polarization optical studies of the structure of nerve cells J Cell and Comp Physiol 12 1, 1938
CHINN, P AND F O SCHMITT On the birefringence of nerve sheaths as studied in cross sections J Cell and Comp Physiol 9 289, 1937
COLE, W H The composition of fluids and sera of some marine animals and of the sea water in which they live J Gen Physiol 23 575, 1940
CORTEGGIANI, E Contribution a l'etude de l'acetylcholine libre et dissimulée sous forme d'un complexe dans le cerveau Thèse, Université de Paris, 1938
CORTEGGIANI, E AND A SERFATY Acétylcholine et cholinestérase chez les insectes et les arachnides C R Soc Biol 131 1124, 1939
COWAN, S L The $CO_2$ dissociation curves and the buffering of crab's muscle and nerve preparations J Exper Biol 10 401, 1933
The action of potassium and other ions on the injury potential and action current in *Maia* nerve Proc Roy Soc B, 115 216, 1934
CRESCITELLI, F AND T L JAHN Oscillatory electrical activity from the insect compound eye J Cell and Comp Physiol 19 47, 1942
CROZIER, W J Reversal of inhibition by atropine in caterpillars Biol Bull 43 239, 1922
CROZIER, W J AND G F PILZ Central nervous excitation and alkaloids in insects Am J Physiol 69 41, 1924
DAVENPORT, D The effects of acetylcholine, atropine and nicotine on the isolated heart of the commercial crab, *Cancer magister* Dana Physiol Zool 14 178, 1941
Further studies in the pharmacology of the heart of *Cancer magister* Dana Biol Bull 82 255, 1942
DAVENPORT, D , J W LOOMIS AND C F OPLER Notes on the pharmacology of the hearts of *Arriolimax columbianus* and *Astacus trowbridgei* Biol Bull 79 498, 1940
DUBUY, H G The electrical phenomena of the crustacean nerve-muscle system Am J Physiol 114 224, 1935
ELLIS, C H , C H THIENES AND C A G WIERSMA The influence of certain drugs on the crustacean nerve-muscle system Biol Bull 83 334, 1942
ENGEL, G L AND R W GERARD The phosphate metabolism of invertebrate nerve J Biol Chem 112 379, 1935
ETS, H N The effect of potassium on the cold blocking of spider crab nerve J Cell and Comp Physiol 8 101, 1936
FENN, W O , P M COBB, A H HEGNAUER AND B S MARSH Electrolytes in nerve Am J Physiol 110 74, 1934
FURUSAWA, K The depolarization of crustacean nerve by stimulation or oxygen want J Physiol 67 325, 1929
GADDUM, J H Pharmacology Oxford Univ Press, 1940
GARREY, W E An analysis of the action of acetylcholine on the cardiac ganglion of *Limulus polyphemus* Am J Physiol 126 182, 1942

BAUDIN, O Recherches sur l'action physiologique des pyréthrines Vigot Frères, Paris, 1937

GOODMAN, L AND A. GILMAN The pharmacological basis of therapeutics New York Macmillan, 1941

GRUNDFEST, H Bioelectric potentials Ann Rev Physiol 2: 213 1940

GUTTMAN, R. Stabilization of spider crab nerve membranes by alkaline earths as manifested in resting potential measurements J Gen Physiol 23: 343, 1940

HAMILTON, H L The action of acetylcholine atropine and nicotine on the heart of the grasshopper (*Melanoplus differentialis*) J Cell and Comp Physiol 13: 91, 1939

VAN HARREVELD A The nerve supply of doubly and triply innervated crayfish muscles related to their funtion J Comp Neurol 70 267 1939a

The motor innervation of a triply innervated crustacean muscle J Exper Biol 16: 398 1939b

Doubly triply, quadruply and quintuply innervated crustacean muscles J Comp Neurol 70: 285 1939c

VAN HARREVELD, A AND C A G WIERSMA. The double motor innervation of the adductor muscle in the claw of the crayfish J Physiol 88 78 1936

The triple innervation of crayfish muscle and its function in contraction and inhibition J Exper Biol 14 448 1937

The function of the quintuple innervation of a crustacean muscle J Exper Biol 16: 121 1939

HARTLINE, H K AND GRAHAM Nerve impulses from single receptors in the eye J Cell and Comp Physiol 1: 277, 1932

HEINBECKER P The potential analysis of a pacemaker mechanism in *Limulus polyphemus* Am J Physiol 117: 686, 1936

HODGKIN A L The subthreshold potentials in a crustacean nerve Proc Roy Soc B 126: 87, 1939

HOFFMAN, P Ueber die doppelte Innervation der Krebsmuskeln, zugleich ein Beitrag zur Kenntnis nervöser Hemmungen Ztschr f Biol 63: 411, 1914

HOLMES, E G Carbohydrates of crab nerve Biochem J 23: 1182 1929

HOLMES W R J PUMPHREY AND J Z YOUNG The structure and conduction velocity of the medullated nerve fibers of prawns J Exper Biol 18: 50, 1941

JASPER, H H AND A M MONNIER. Pseudo-chronaxies des systèmes neuro musculaires des crustacés dues a la résponse rhythmique du nerf C R. Soc Biol 112 233, 1933

KATZ B Neuro muscular transmission in crabs J Physiol 87: 199, 1936a

Multiple response to constant current in frog s medullated nerve J Physiol 88: 239, 1936b

KEIGHLY, G AND C A G WIERSMA Heat production of slow and fast contractions of a crustacean muscle with double motor innervation J Cell and Comp Physiol 17 305 1941

KNOWLTON F P Observations on the dual contraction of crustacean muscle Biol Bull 82: 207 1942

HEILBRUNN L. V An outline of general physiology 2nd ed , Saunders, 1943

KNOWLTON F P AND C J CAMPBELL. Observations on peripheral inhibition in arthropods Am J Physiol 91: 10 1929

LACROIX, P Recherches cytologiques sur les centres nerveux chez les invertebres II *Astacus fluviatilis* La Cellule, 44 253 1935

LEVIN, A Fatigue retention of action current and recovery in crustacean nerve J Physiol 63: 113 1927

LUCAS, K. On summation of propagated disturbances in the claw of *Astacus*, and on the double neuro-muscular system of the adductor J Physiol 51: 1 1917

LULLIES H Aktionsströme und Fasergruppen in Extremitatennerven von *Maja squinado* Pflüger s Arch 233: 584, 1933

MACINTOSH, F C The distribution of acetylcholine in the peripheral and the central nervous system J Physiol 99 436, 1941

MACLEAN, M N AND A B L BEZNAK The effect of sympatheticomimetic and para sympatheticomimetic drugs upon the hearts of decapod Crustacea Magyar biológiai kutato intezet Munkai 6 258, 1933

MANGOLD, E Untersuchungen über die Endigungen der Nerven in den quergestreiften Muskeln der Arthropoden Ztschr f allg Physiol 5 135, 1905

MARCU, O Nervenendigungen an den Muskelfasern von Insecten Anat Anz 67: 369, 1929

MARMONT, G The effect of chemical agents on the "local response" of large single crustacean axons Am J Physiol , 133 376, 1941

MARMONT, G AND C A G WIERSMA On the mechanism of inhibition and excitation of crayfish muscle J Physiol 93: 173, 1939

MARNAY, A AND D NACHMANSOHN Cholinestérase dans le nerf de homard C R Soc Biol 125: 1005, 1937

MEANS, O W , JR Cholinesterase activity of tissues of adult *Melanoplus differentialis* (Orthoptera, Acrididae) J Cell and Comp Physiol 20 319, 1942

MEYERHOF, O AND W SCHULZ Über die Atmung des marklosen Nerven Biochem Ztschr 206 158, 1929

MIKALONIS, S J AND R H BROWN Acetylcholine and cholinesterase in the insect central nervous system J Cell and Comp Physiol 18 401, 1941

MONAGHAN, B R AND F O SCHMITT The absorbtion spectrum of medullated and non medullated nerves Proc Soc Exper Biol and Med 28 705, 1931

MONNIER, A M AND M DUBUISSON Etude a l'oscillographe cathodique des nerfs pedieux de quelques arthropodes Arch Inter Physiol 34 25, 1931a

NACHMANSOHN, D AND H M JOHN Studies on choline acetylase I Effect of amino acids on the dialyzed enzyme Inhibition by $\alpha$-Ketoacids J Biol Chem 158 157, 1945

NACHMANSOHN, D AND M A ROTHENBERG Studies on cholinesterase I On the specificity of the enzyme in nerve tissue J Biol Chem 158 653, 1945

OCHOA, S Enzymic phosphorylations in invertebrate muscle Biochem J 32 237, 1938

PANTIN, C F A On the excitation of crustacean muscle I J Exper Biol 11 11, 1934
On the excitation of crustacean muscle II Neuromuscular facilitation J Exper Biol 13 111, 1936a
On the excitation of crustacean muscle III Quick and slow responses J Exper Biol 13 148, 1936b
On the excitation of crustacean muscle IV Inhibition J Exper Biol 13 159, 1936c

PATERSON, E K , M I DRUMM AND A G RICHARDS, JR Lipids in the central nervous system of the honey bee Arch Biochem 1 201, 1945

PIOTROWSKI, G On the muscle nerve physiology of the crayfish especially with regard to inhibition J Physiol 14 163, 1893

PRINGLE, J W S Proprioception in insects I A new type of mechanical receptor from the palps of the cockroach J Exper Biol 15 101, 1938a
Proprioception in insects II The action of the campaniform sensilla on the legs J Exper Biol 15 114, 1938b
Proprioception in insects III The function of the hair sensilla at the joints J Exper Biol 15 467, 1938c
The motor mechanism of the insect leg J Exper Biol 10 220, 1939

PROSSER, C L Action potentials in the nervous system of the crayfish I Spontaneous impulses J Cell and Comp Physiol 4 185, 1934a
Action potentials in the nervous system of the crayfish II Responses to illumination of the eye and caudal ganglion J Cell and Comp Physiol 4 363, 1934b

Action potentials in the nervous system of the crayfish III Central responses to proprioceptive and tactile stimuli J Comp Neurol 62: 494 1935a
Action potentials in the nervous system of the crayfish IV Effect of tempera ture on nerve impulses arising "spontaneously in abdominal ganglia. J Gen Physiol 19 65, 1935b
Action potentials in the nervous system of the crayfish V Temporal relations in presynaptic and postsynaptic responses J Cell and Comp Physiol, 16: 25 1935c
Evidence for chemical control of spontaneous ' activity of isolated ganglia Am J Physiol 123: 165 1938
Action potentials in the nervous system of the crayfish Effects of drugs and salts upon synaptic transmission J Cell and Comp Physiol 16: 25, 1940
An analysis of the action of acetylcholine on hearts, particularly in arthropods Biol Bull 83 145, 1942
Single unit analysis of the heart ganglion discharge in *Limulus polyphemus* J Cell and Comp Physiol 21: 295 1943a
An analysis of the action of salts upon abdominal ganglia of crayfish J Cell and Comp Physiol 22: 131 1943b

Prosser, C L and C C Buehl Oxidative control of spontaneous activity in the nervous system of the crayfish. J Cell and Comp Physiol 14: 287, 1939

Pumphrey, R. J and A F Rawdon-Smith Hearing in insects the nature of the response of certain receptors to auditory stimuli Proc Roy Soc B 121: 18, 1936
Synaptic transmission of nervous impulses through the last abdominal ganglion of the cockroach Proc Roy Soc B 122 106, 1937

Raper, H S The tyrosine tyrosinase reaction V Production of *l*-3-4-dihydroxyphen ylalanine from tyrosine Biochem J 20: 735 1926

Retzius, G Ueber myelinhaltige Nervenfasern bei Evertebren. Biol Fören. Förhand 1: 58 1888
Zur Kenntnis des Nervensystems der Crustacean. Biol Untersuch 1: 1, 1890

Richards A G Jr. Lipid nerve sheaths in insects and their probable relation to in secticide action. J N Y Ent Soc 51 55 1943
The structure of living insect nerves and nerve sheaths as deduced from the op tical properties J N Y Ent Soc 52: 285, 1944

Richards, A G Jr and J L Weygandt The selective penetration of fat solvents into the nervous system of mosquito larvae J N Y Ent Soc 53 153, 1945

Rijlant, P Tonus et contraction musculaire chez un Arthropode (Scorpion) C R. Soc Biol 115: 1415 1934

Roeder, K D Synchronized activity in the optic and protocerebral ganglia of the grass hopper *Melanoplus femur rubrum* J Cell Comp Physiol 14: 299 1939
The effect of K and Ca on the spontaneous activity of the isolated crayfish nerve cord J Cell and Comp Physiol 18: 1 1941

Roeder K D and S Roeder. Electrical activity in the isolated ventral nerve cord of the cockroach I The action of pilocarpine nicotine eserine and acetylcholine J Cell and Comp Physiol 14: 1, 1939

Schaller W Action of potassium on bound acetylcholine in lobster nerve cord J Cell and Comp Physiol 26 15 1945

Schmitt, F O and R. S Bear The optical properties of vertebrate nerve axons as re lated to fiber size J Cell and Comp Physiol 9: 261 1937

Schmitt, F O R. S Bear and R. H Silber. Organic and inorganic electrolytes in lobster nerves J Cell and Comp Physiol 14: 351 1939

Shepard, H H The chemistry and toxicology of insecticides Burgess Publ Co, Minne apolis, Minn. 1939

Silber, R. H The free amino acids of lobster nerve J Cell and Comp Physiol 18: 21, 1941

SILBER, R H AND F O SCHMITT The rôle of free amino acids in the electrolyte balance of nerve J Cell and Comp Physiol 16 247, 1940

SMITH, R I Acetylcholine in the nervous tissue and blood of crayfish J Cell and Comp Physiol 13 335, 1939

SOLANDT, D Y The measurement of "accommodation" in nerve Proc Roy Soc B 119 355, 1936

TAHMISIAN, T N Enzymes in ontogenesis Cholinesterase in developing *Melanoplus differentialis* eggs J Exper Zool 92 199, 1943

TAYLOR, G W The optical properties of the shrimp nerve fiber sheath J Cell and Comp Physiol 18 233, 1941

The correlation between sheath birefringence and conduction velocity with special reference to cat nerve fibers J Cell and Comp Physiol 20 359, 1942

WATERMAN, T H A comparative study of the effects of ions on whole nerve and isolated single nerve fiber preparations of crustacean neuromuscular systems J Cell and Comp Physiol 18 109, 1941

WELSH, J H Chemical mediation in crustaceans I The occurrence of acetylcholine in nervous tissues and its action on the decapod heart J Exper Biol 16 198, 1939a

Chemical mediation in crustaceans II The action of acetylcholine and adrenalin on the isolated heart of *Panulirus argus* Physiol Zool 12 231, 1939b

Chemical mediation in crustaceans IV The action of acetylcholine on isolated hearts of *Homarus* and *Carcinides* J Cell and Comp Physiol 19 271, 1942

WELSH, J H AND H H HASKIN Chemical mediation in crustaceans III Acetylcholine and autotomy in *Petrolisthes armatus* (Gibbs) Biol Bull 76 405, 1939

WELSH, J H AND J E HYDE The effects of potassium on the synthesis of acetylcholine in brain Am J Physiol 142 512, 1944a

Acetylcholine content of the myenteric plexus and resistance to anoxia. Proc Soc Exper Biol and Med 55 256, 1944b

WENSE, T Über den Nachweis von Adrenalin in Würmen und Insekten *Pflüger's* Arch 241 284, 1938

WIERSMA, C A G The inhibitory nerve supply of the leg muscles of different decapod crustaceans J Comp Neurol 74 63, 1941

WIERSMA, C A G AND C H ELLIS A comparative study of peripheral inhibition in decapod crustaceans J Exper Biol 18 223, 1942

WIERSMA, C A G AND A VAN HARREVELD A comparative study of the double motor innervation in marine crustaceans J Exper Biol 15 18, 1938a

The influence of the frequency of stimulation on the slow and fast contraction in crustacean muscle Physiol Zool 11 75, 1938b

The interactions of the slow and fast contractions of crustacean muscle Physiol Zool 12 43, 1939

WIERSMA, C A G AND R G HELFER The effects of peripheral inhibition on the muscle action potentials of the crab Physiol Zool 14 296, 1941

WIERSMA, C A G AND G MARMONT On the motor and inhibitory innervation of the legs of brachyuran decapods Physiol Zool 11 312, 1938

WIERSMA, C A G AND E NOVITSKI The mechanism of the nervous regulation of the crayfish heart J Exper Biol 19 255, 1942

WIGGLESWORTH, V B The storage of protein, fat, glycogen and uric acid in the fat body and other tissues of the mosquito larvae J Exper Biol 19 56, 1942

The principles of insect physiology New York, E P Dutton, 1939

YEAGER, J F AND J B GAHAN Effects of the alkaloid nicotine on the rhythmicity of isolated heart preparations from *Periplaneta americana* and *Prodenia eridania* J Agric Res 55 1, 1937

YEAGER, J F AND S C MUNSON Physiological evidence of a site of action of DDT in an insect Science 102 305, 1945

YOUNG, J Z The structure of nerve fibers in cephalopods and crustacea Proc Roy Soc B 121 319, 1936

PHYSIOLOGICAL REVIEWS
VOL. 26 OCTOBER, 1946 No 4

# BIOTIN AND THE AVIDIN BIOTIN COMPLEX

ROY HERTZ

*U S Public Health Service National Institute of Health Bethesda Md*

The present review is an attempt to bring together the available biological information regarding biotin and the avidin biotin complex. The chemical aspect of this subject has been adequately treated in several recent reviews and will not be dealt with here (1, 2, 3)

Wildiers (4) first appreciated the requirement of certain strains of yeast for a trace factor, which he called "bios" Subsequent study indicated that the term "bios" referred to multiple factors affecting yeast growth One of these factors was isolated by Kögl and Tonnis (5) and named "biotin"

Avidin is the substance which is considered to be responsible for the noxious effects of the ingestion of large amounts of egg white in several species of animals (6) It has also been called "anti biotin" (7), but general usage has made avidin the more acceptable term, particularly since other substances having anti biotin activity have been described (133), (20), (20a), (20b)

*Methods of assay for biotin* In view of the quantitative requirement of numerous microorganisms for biotin, microbiological tests for this substance have been highly developed Table 1 summarizes the essential features of the more commonly used tests They all depend upon the same principle. The test organism is grown on a medium that will afford increasing bacterial growth as additional amounts of biotin are added Optimal growth must be achieved upon the addition of an excess of biotin The growth obtained may be measured turbidimetrically or acidimetrically

The preparation of the sample for assay remains one of the most difficult steps in testing for this potent growth factor Lampen et al (17) have emphasized the occurrence of biotin in bound form in most natural materials Various methods have been devised for the release of bound biotin since the vitamin is available to the test organism only in the free state Autolysis, enzymatic digestion, and acid hydrolysis are the chief methods thus far employed, and none of them is universally applicable (8, 13, 16) Incomplete liberation by too mild a treatment and possible destruction by more drastic procedures must be carefully avoided The shortcomings of such extraction procedures are reflected in the widely differing assay results reported for many tissues and foods (8, 11, 12, 17)

Interpretation of the microbiological results is further complicated by the existence of several biotinlike factors which support the growth of the several test organisms to varying degrees Burk and Winzler have referred to these growth factors as "vitamers" and have indicated differences both in their heat stability and in their respective potencies for several organisms (18) Moreover, the diaminocarboxylic acid derived from biotin, the natural occurrence of which

is totally unknown, has been shown to have 10 per cent of the yeast-growth activity of biotin itself (19) A considerable series of biotin derivatives, which are discussed later, have been shown to have distinctive potencies for several different test organisms (20, 20a, 20b, 124–128a) Accordingly, a microbiological determination of biotin in a natural material is merely a resultant of numerous growth effects and represents only that proportion of the total complex of biotin and biotinlike substances which has been rendered available to the test organism employed

Animal assays for biotin have been largely neglected because of the greater convenience and sensitivity of the microbiological methods However, Gyorgy and his associates (21–23) successfully employed the egg-white-injured rat as a test animal in their earlier studies of the properties of vitamin H In addition, Ansbacher and Landy (24) prepared a deficiency-producing diet for the chick

TABLE 1

*Microbiological methods for biotin assay*

| TEST ORGANISM | RANGE IN MILLI MICROGRAMS | METHOD OF READING | ERROR | REFERENCE |
|---|---|---|---|---|
| | | | *Per cent* | |
| Saccharomyces cervisiae | 0 002–1 0 | Turbidimetric | | (8) |
| Saccharomyces cerevisiae no 139 | 1 0–0 5 | Turbidimetric | 5 | (9) |
| Lactobacillus arabinosus | 0 25–1 5 | Acidimetric | | (10) (11) |
| Lactobacillus casei | 40–1000 | Acidimetric | 10 | (12) (14) |
| Lactobacillus casei | 100–300 | Acidimetric | | (13) |
| Clostridium butylicum | 0 01–? | Turbidimetric | 20 | (15 17 |
| Rhizobium trifolii | 0 016–? | Turbidimetric | | 61 |

which could be used for assay purposes A study of the comparative biological potency of natural material containing biotin as determined by the available animal and microbiological methods would probably afford some information regarding the various forms in which biotin may occur in nature Similar studies in relation to pyridoxine and folic acid have led to the recognition of the several forms in which these vitamins may be found (25, 26)

*Occurrence of biotin* In spite of the sensitivity of the microbiological tests, our knowledge of the actual distribution of biotin in nature is not precise The difficulties of adequately extracting the entire biotin content from tissues and foods have led observers to accept the highest values obtained as the true measure of biotin content For the reasons already discussed, these determinations can be regarded as only gross approximations until more is known concerning the conditions for optimal release of biotin from the bound form Several tabulations of such determinations of biotin in a wide variety of natural materials are available (8, 11, 12, 16–18) Biotin is found in varying degree in almost all substances

tested The high content of biotin in liver and yeast indicates its close relation ship to the B-complex, an observation actually made by Boas (27) in her early studies on egg white injury

The presence of biotin in several highly purified natural materials warrants special consideration Hoagland et al (28) have shown that the elementary bodies of vaccinia contain both free and bound biotin in approximately the same amount as that found in such rich sources as egg yolk and liver Spruce and Schoenbach (29) reported that highly purified concentrates of the tobacco mosaic virus protein contained no biotin Miller et al (30a) found considerable amounts of bound biotin in numerous preparations of crystalline enzymes, but the concentrations exceeded by far the expected amount of biotin assuming an equimolar ratio of biotin to protein This suggested that the demonstrated biotin is presumably not an integral part of the enzyme molecule Williams et al (30) found that biotin as well as the other B-complex factors were present in numerous preparations of presumably pure proteins, including several crystalline enzymes and viruses. The data do not determine whether biotin is an integral part of these substances or is simply present as a trace contaminant In any event, the microbiological determination of biotin and the other B-complex factors may be utilized as a highly sensitive indicator of the purity of such biolog ical preparations (30a)

The occurrence of biotin in numerous phyla has been reported by Woods et al (31) This almost universal distribution of biotin further emphasizes its basic biological significance

*Biotin and avidin in nutrition and reproduction.* Bond (32) reported that rats failed to grow when fed a diet containing 30 per cent dried egg white.

In 1924 Boas called further attention to the peculiar nutritive properties of dried egg white (33, 34) Her thorough description of the observable features of egg white injury in the rat includes virtually all that has been noted up to the present time.

The rats grow well and are usually in good health for from 2 to 3 weeks Then red scaly patches appear at the corners of the mouth, the coat becomes rough and sticky and the long hairs fall out The fur on the abdomen shows at first a characteristic ribbed appearance followed by the development of bald areas Meanwhile the red patches spread to other parts of the body and the picture is one of an eczematous dermatitis There are even skin hemorrhages in severe cases The region round the mouth is always the most severely affected though there is often such marked blepharitis that the eyes are closed. The loss of hair is often extensive In a few cases oedema of the feet has been seen but this does not usually occur These rats always have a distinctive, somewhat musty smell probably due to some constituent of the urine The body weight remains stationary for a week or two, but falls slowly during the second stage of the disease This is reached about 2 to 3 weeks after the development of the first signs of deficiency To the dermatitis symptoms of nervous upset are now added There is pronounced spasticity of the limbs particularly of the hind legs and the back is arched The rat assumes in many cases a kangaroo-like posture Some of the rats do not show marked spasticity but assume a crouching atti tude and display a curious swimming movement with the front paws Death which occurs in the final phase, is preceded by a rapid loss of weight and the animal shows signs of ex treme cyanosis. Rigor mortis sets in rapidly Post mortem there is an almost complete absence of fat and the skin is infiltrated and vascularized.

The ability of certain foods to protect the rat against egg white injury was attributed by Boas to a "protective factor" (34) She concluded that the drying process rendered egg albumin toxic (34) Friedberger et al (35, 36) claimed that in rats fed cooked eggs the toxic effects were more marked and occurred earlier than in those fed raw eggs Contrarily, Scheunert et al (37, 38) and Stenquist (39) reported a marked toxic effect in rats fed raw eggs and normal growth in those receiving cooked eggs The basis for the discrepancy between these authors' findings has not been determined

Findlay and Stern (50) also described egg white injury in the rat and suggested its similarity to "pink disease" in children

Parsons and Kelly (40, 41) demonstrated the heat lability of the toxic factor in egg white and found that it persisted in egg white denatured by alcohol and leached in running water The toxic effects were reversed by feeding liver or kidney and less readily by dried milk, wheat germ, dried yeast, and dried egg yolk (41) Salmon and Goodman (42) also described the toxic effects of feeding rats egg white, either fresh or dried Lease, Parsons and Kelly described egg white injury in the chick, rabbit, monkey and guinea pig as well as in the rat (43) Further studies by Parsons and her associates extended our knowledge of the anti-egg-white-injury factor and of its distribution in various foods (44–49)

Boas (27) called attention to the similar distribution of the "protective factor X" and the factors of the B-complex Gyorgy (51, 52) emphasized the vitaminlike character of this factor by referring to it as "Vitamin-H", a term derived from the German word "Haut" for skin This concept guided the extended studies of Gyorgy and his associates concerning the chemical character and natural distribution of vitamin H (21–23, 56) This increasing body of descriptive and quantitative data led to the ultimate proof of the identity of vitamin H and biotin (57, 58, 59)

Nilsson et al (60) and West and Wilson (61) meanwhile had suggested from comparative quantitative data on potent concentrates that coenzyme R (62) is identical with biotin This was subsequently substantiated by studies reported by du Vigneaud and his associates (1)

The mechanism whereby egg white injury induces a biotin deficiency has been the subject of several investigations Parsons et al (63) found that the feces of rats suffering from egg-white injury proved curative of this injury only after heating They "suggested as a tentative hypothesis that raw egg white derives its essential capacity to produce a pathological condition not from its own possible lack of the protective factor in available form nor from the presence of a hypothetical anti-enzyme but from its characteristic in the digestive tract of combining with and holding in an unabsorbable form the protective factor which originated either from the diet alone or, also from an excretion into the digestive tract" Eakin et al (64) showed that an egg-white-injury-producing diet for chicks contained even more biotin than did a control diet containing casein, but that the tissues of chicks maintained on the former contained less biotin than the controls They concluded that egg-white injury is attributable to the capacity of egg white to render the dietary biotin unavailable to the tissues

of the body Eakin et al (65) found further that a constituent of raw egg white is capable of rendering biotin unavailable to yeast cells in vitro, and that heating effected a release of the biotin for the support of yeast growth Gyorgy et al (66) then showed a direct correlation between the capacity of an egg white preparation to produce egg white injury in the rat and its effectiveness in combining with biotin in vitro The active constituent of egg white has accordingly been called "avidin" which is a contracted form of the original "avid albumin", a term signifying its active affinity for biotin (6)

The nature of avidin and the character of the combination of avidin with biotin have been the subject of considerable study Gyorgy and Rose (67) observed that when avidin concentrates were administered parenterally they had a decisive therapeutic effect upon egg white injury This effect was attributed to the bound biotin content of the avidin concentrates which presumably became available after injection Gyorgy, Rose and Tomarelli (68) reported the sensitivity of avidin to ultraviolet light and to acidification at pH 1.8, calling particular attention to the fact that the presence of the avidin biotin complex tended to stabilize any excess avidin present during such acidification Gyorgy and Rose (69) further found that the avidin biotin complex resists treatment with proteolytic enzymes including pepsin trypsin, pancreatin and papain In addition, incubation with preparations of liver, kidney, muscle or blood had no effect. However, about 10 to 20 per cent of the biotin could be liberated in a form which was dialyzable and would support yeast growth by treatment at pH 3 with 0 45 per cent $H_2O_2$ These workers therefore feel that the metabolism of biotin and avidin is more intimately related to oxidation reduction systems than to digestive enzymes

It should be emphasized that not all substances capable of supporting yeast growth in the absence of biotin are avidin-combinable Thus, du Vigneaud et al (70) prepared a diamino-carboxylic acid from biotin which supports yeast growth but is not inhibited by avidin, indicating that the urea ring is presumably essential for the formation of the avidin biotin complex. Dittmer and du Vigneaud (20a) have shown that shortening of the side chain of desthiobiotin eliminates the avidin-combining power of the derived compounds Oppel (71) detected the presence of an avidin uncombinable substance in the urine of the dog rabbit, rat and man. Burk and Winzler (18) have determined the relative proportion of yeast supporting activity of various natural materials which is not combinable with avidin Chu and Williams have employed different testing conditions and conclude that avidin uncombinable factors are found only in urine (18a)

Woolley and Longsworth (7) pointed out that if yeast cells are permitted exposure to a biotin-containing medium for 6 hours, the subsequent addition of avidin would be without effect upon their further growth They interpreted this phenomenon as indicating that the absorption of the biotin into the yeast cell rendered it unavailable to the avidin Hertz (72) has confirmed this observation and has found in addition that the acid-extractable biotin content of a 6 hour yeast culture remains avidin-combinable

Some information concerning the chemical character of avidin has been ac

quired Parsons and Kelly (41) found that saturation with ammonium sulfate precipitated the active material and that it was absent from purified ovalbumen Eakin et al (65, 73) prepared potent concentrates by ammonium sulfate fractionation of the acetone precipitable portion of fresh egg white Pennington, Snell and Eakin (74) prepared biologically active crystals, the chemical behavior of which indicated that the "substance may be a protein with a large carbohydrate moiety"

Woolley and Longsworth (7) derived from raw egg white an amorphous fraction which they regarded as a single substance, as evidenced by homogeneity in electrophoresis and in sedimentation determinations The substance was 15,000 times as active as egg white and proved to be a basic protein with an isoelectric point at pH 10, and an estimated molecular weight of 70,000 Curiously, the pure substance retained activity after boiling in dilute acetic acid and was stable at room temperature in solution at a pH ranging from 1 0 to 11 0

The distribution of avidin in nature has been studied to a limited extent Gyorgy and Rose (75) determined that the amount of avidin present in the average hen's egg is sufficient to more than completely neutralize its biotin content Woolley and Longsworth (7) detected traces of anti-biotin activity in certain purified basic proteins Hertz and Sebrell (76) found relatively high avidin titres in the eggs of not only other birds such as the turkey, duck and goose, but also in the egg jelly of two species of frogs They emphasized the fact that the egg albumen represents a secretion from the mucosal lining of the oviduct and demonstrated the presence of avidin in the oviduct tissue of the hen and frog (76)

Fraps, Hertz and Sebrell (77) found that avidin is present only in the albumen-secreting portion of the oviduct Moreover, the oviduct of the nonlaying hen was free of avidin, indicating that avidin production may depend upon ovarian function Hertz, Fraps and Sebrell (78, 79) further demonstrated that avidin secretion could be induced in the oviduct of the newborn chick by combined treatment with stilbestrol and either progesterone, desoxycorticosterone acetate, or testosterone propionate Hertz (72) has tested mucosal scrapings from the oviducts of pig, cow and guinea pig for avidin with entirely negative results

The suggested rôle of avidin and biotin in the physiology of reproduction remains unclarified, but several additional studies in this connection should be cited Cravens et al (80) found that the low hatchability of eggs from hens maintained on a low-biotin diet could be overcome by adding biotin-containing concentrates to the diet Kennedy and Palmer (81) have shown that egg-white injury in the rat does not interfere with conception but leads to frequent fetal resorption and impaired lactation Snell and Quarles (82) showed that the biotin content of the hen's egg remains almost constant throughout incubation Singher et al (83) found that the liver of the biotin-deficient rat retains its capacity to inactivate estradiol in vitro These studies are as yet too diverse to permit any generalizations to be made from them

The possible relationship between avidin and other biologically active principles found in egg white has been investigated Laurence (53) reported a remark-

able correspondence between the lysozyme activity and avidin potency of seven egg white concentrates Meyer (54) reported similar results and stated further that the addition of biotin markedly enhances the lysozyme activity of egg white preparations However, Landy (55) had previously indicated a lack of correlation between lysozyme and avidin activity Woolley and Longsworth (7) also noted that their pure avidin preparations lacked lytic power Alderton et al (84) reported that pure lysozyme not only failed to inactivate biotin but also that the addition of biotin did not enhance its potency In addition, highly purified avidin concentrates showed no lysozyme activity The more cogent evidence indicates, therefore, that avidin and lysozyme are distinct substances and that biotin is not involved in the lysozyme effect

Biotin is known to be required by numerous species in addition to the rat Norris and Ringrose (85) produced a dermatitis in chick on both a purified diet and one containing egg white Lease and Parsons (47) also observed a marked dermatitis in chicks fed egg white This chick dermatitis was more specifically attributed to biotin deficiency by Hegsted et al (86), who were able to cure severe skin lesions in chicks fed a purified diet by feeding small amounts of biotin concentrates Ansbacher and Landy (24), employing a heated ration, were able to cure the deficiency syndrome by administering crystalline biotin Hegsted et al (87) subsequently reported similar results with pure biotin.

Perosis has been attributed to a biotin deficiency in both chicks (88) and turkey poults (89) Richardson, Hogan and Miller (90) have indicated that perosis in chicks may be a nonspecific effect of several nutritional deficiencies in addition to biotin deficiency Similar conclusions were reached by Patrick et al (91) regarding perosis in turkey poults

Among the rodents, the golden hamster has been shown to require biotin for optimal growth and reproduction (92) However, the cotton rat is able to grow on a highly purified diet without added biotin (93) Reference has already been made to the observations of Lease et al upon egg white injury in the rabbit and guinea pig (43)

Man and the *Macaca rhesus* monkey are the only primates whose biotin requirement has been studied Lease, Parsons and Kelly observed a characteristic dermatitis and general debility in monkeys fed a diet containing egg white (43) Waisman and Elvehjem noted a beneficial effect of biotin upon the hair coat and skin of monkeys maintained on a purified diet (94)

Oppel (71, 95, 96) has determined the urinary and fecal excretion of biotin in man under varying conditions The normal adult excreted in the urine from 7 to 89 micrograms of biotin daily Ingestion of a crude biotin concentrate was promptly reflected in a marked rise in urinary output No correlation could be made between any of the numerous disease conditions studied and the urinary output of biotin The fecal excretion of biotin was independent of the dietary biotin and exceeded it in most instances The occurrence in human urine of an avidin-uncombinable substance which will support yeast growth has already been mentioned (71, 18)

Gardner et al (96a) followed the biotin excretion in 3 normal young women on

a milk diet The urinary biotin ranged from 40 to 57 micrograms daily and the fecal biotin was in the same range In two of the individuals the output exceeded the intake [1] These findings are in agreement with those of Oppel (96)

Sydenstricker et al (97, 98) fed an egg-white-containing diet to volunteers for prolonged periods of time After 3 to 4 weeks a transient dermatitis was observed After 5 weeks the following clinical changes were noted lassitude, somnolence, muscle pains, hyperesthesia and anorexia Administration of a biotin concentrate, in amounts ranging from 150 to 300 micrograms per day relieved the patients of these symptoms in 3 to 5 days The urinary excretion level of these patients fell gradually while they were on the experimental diet and rose abruptly upon the administration of the biotin concentrate

Williams (99) presented a detailed case report of an individual presenting a dermatitis associated with a history of having subsisted on raw eggs and wine for many years The dermatitis cleared while the patient was on a hospital diet but could not be reproduced on a diet of raw eggs, presumably because of biotin contributed by organisms harbored in the individual's infected urinary tract The case is an interesting clinical curiosity but is not to be considered a proved instance of human biotin deficiency

Fraenkel and Blewett showed that the larvae of the beetle, *Tribolium confusum*, require biotin when grown on an artificial diet (100, 101, 102) Rosenthal and Reichstein reported similar observations on the same organism (103)

The microscopic pathology from egg-white injury in the rat has been described in a series of studies by Sullivan et al (104, 105, 106) In the skin, hyperkeratosis, parakeratosis, acanthosis and edema were observed The nervous system was found to show no change, and the hypertonicity was considered to be associated with pathological muscle changes resembling those seen in vitamin E deficiency Shaw and Phillips (107) also reported no changes in the nervous system of egg-white-injured rats and described the muscles as grossly small and pale, with microscopic evidence of atrophy but not of true degeneration

McHenry and Gavin (108) found that a liver fraction which was rich in biotin produced fatty livers in rats Subsequent study (109) showed that pure biotin was also effective and that inositol and lipocaic both counteracted this type of fat deposition (110) Later, MacFarland and McHenry (110a) reported that the biotin type of fatty liver which had been previously characterized as resistant to the lypotropic action of choline was in fact only partially resistant to choline The more complete choline-resistance induced by the administration of a liver-fraction was therefore attributed to a factor other than biotin and this special type of fatty liver was shown to be peculiarly responsive to inositol. Best et al (110b) have concluded that the response to inositol and choline of the "biotin fatty liver" is not distinctive in character Moreover, the apparent greater responsiveness of the liver-fraction-induced-fat to inositol is regarded by them as a synergistic lypotropic effect of inositol with the choline known to be contained

[1] Gardner, J , Parsons, H T and Peterson, W H (Arch Biochem 8, 339, 1945) have extended these studies in ten subjects with similar results

in the liver fraction employed This interpretation seems to account more adequately for the data presented

The effects of biotin on other specific tissues have received some study Fischer (111) noted no effect of excess biotin either upon the rate of atrophy of the denervated gastrocnemius muscle or upon the return of function following crushing of the sciatic nerve in the rat Lazere et al (112) similarly reported no effect of biotin deficiency in the rat upon the usual results of crushing the tibial nerve Hamilton and Plotz (113) found that 65 per cent of explants of nerve tissue grown in vitro showed stimulating effects of biotin when added to a medium containing plasma, embryonic extract and Tyrode solution Contrarily, Burt (114) observed no growth-stimulating effect of biotin on nerve cells from chick embryos in tissue culture

Boas (27) and numerous other observers have noted neurological changes in the hind limbs of rats subjected to egg white injury Nielsen and Elvehjem (115) reported a prompt relief of the paralysis exhibited by biotin-deficient rats following biotin administration Smith (116) has described a progressive paralysis in dogs maintained on a purified diet supplemented with eight members of the B-complex and excluding biotin Prodromal features of the paralysis include loss of co-ordination, weakness, and a transient generalized paralysis from which the animal recovers The final paralysis is cured in a few hours by administration of biotin in doses as low as 100 micrograms per kilogram The syndrome is also characterized by cardiac failure which in some cases leads to sudden death [1]

Schmidt and Landy's completely negative findings on the effect of biotin on the blood pressure, heart rate, and respiration of anesthetized cats indicate that biotin lacks marked pharmacodynamic effect in the normal animal (117)

The influence of biotin upon the susceptibility to and the course of infection has been studied to a limited extent Trager (118, 119) found that biotin deficient chicks and ducks exhibit an increased susceptibility to certain strains of malaria, as evidenced either by a higher peak parasite count or a prolonged persistence of high parasite counts in the peripheral blood Moreover, the biotin level of the red blood cells and of the plasma of *P lophurae* infected chicks and ducks rose as the infection advanced and fell as the infection subsided In ducks this effect preceded any increase in circulating young red cells. Control birds suffering from severe pantothenic acid deficiency showed no alteration in the character of the disease following inoculation with *P lophurae* These findings were interpreted as indicating a specific rôle for biotin in the resistance of birds to malarial infection This conclusion received considerable support from confirmatory data of Seeler, Ott and Gundel (119a), who reported that chicks on an egg white diet showed significantly higher parasite counts after inoculation with *P lophurae* than did control chicks receiving the same diet plus biotin

Caldwell and Gyorgy (120) reported a significant prolongation of the duration

[1] Reugamer W R Elvehjem, C A and Hart, E B (Proc Soc Exp Biol & Med 61, 234 1946) have presented evidence indicating that this syndrome may be attributable to potassium deficiency

of infection with *Trypanosoma lewisi* in the biotin-deficient rat This effect could be reversed by the administration of biotin even during the course of the disease

No effect was noted upon the susceptibility to either Lansing strain poliomyelitis or to Theiler's encephalomyelitis of mice rendered deficient in either biotin, pyridoxine or inositol (120a)

Although reference has already been made to existing reviews covering the chemistry of biotin (1, 2, 3), mention should be made of the recently accumulated data concerning the biological effects of compounds closely related to biotin Emerson (121) employed a therapeutic test in the egg-white-injured rat and found that *dl*-biotin was half as active as biotin and that *l*-biotin was inactive when fed at 7 5 times the level of biotin In addition, *dl*-allobiotin, the diaminocarboxylic acid, *dl*-diamino acid, *dl*-allodiamino acid, and *dl*-epiallo-diamino acid derivatives were all inactive at high levels Ott (122) found that *l*-biotin and *dl*-allobiotin were without effect in preventing dermatitis, perosis, and growth failure in chicks fed a purified diet containing 15 per cent dried egg white In rats rendered biotin deficient by feeding either egg white or succinylsulfathiazole, *dl*-desthiobiotin is reported to have only 0 1 to 0 01 per cent activity in comparison with *d*-biotin (123)

Duschinsky et al (124) and Hofmann (125) independently synthesized a biotin analog in which the sulfur was replaced by oxygen. This compound, *dl*-hexhydro-2-oxo-1-furo [3,4] imidazole-4-valeric-acid, was named oxybiotin by the latter group (125) and *o*-heterobiotin by the former (124) Pilgrim et al (126) stated that this compound in *dl*-form is half as active as *d*-biotin for *Lactobacillus arabinosus* and 40 per cent as active as *d*-biotin for *L casei* For *S cerevisiae*, this compound proved 25 per cent as active as *d*-biotin at half maximum growth, but only 8 per cent as active at maximum growth Hofmann et al reported further that this compound possesses therapeutic potency in egg-white injury in rats and both therapeutic and prophylactic effectiveness in biotin deficiency in chicks (128) Rubin et al (127) reported similar findings except that the activity for *Lactobacillus casei* was found to be only 25 per cent in comparison with *d*-biotin In addition, this growth effect for *L casei* was found to be inhibited by desthiobiotin and inferential evidence was presented indicating that the utilization of this compound by yeast involves its conversion to biotin or to a compound of similar activity for yeast growth They also estimated this substance to be 5 per cent as active as *d*-biotin in curing egg-white injury in rats and showed that it combines with avidin in vitro (127)

Hofmann and Winnick (128a) developed a differential test for oxybiotin as opposed to biotin based on the sensitivity of the latter to treatment with dilute potassium permanganate By means of this test they showed that *Saccharomyces cerevisiae* and *Rhizobium trifolii* utilize oxybiotin without converting it to a compound which is sensitive to permanganate treatment Control cultures grown on biotin or on desthiobiotin contain only a permanganate-sensitive substance The basis for the discrepancy between these findings and the data of Rubin et al (127) indicating a conversion of oxybiotin to biotin by yeast cells remains obscure

Additional studies concerning the effects of biotin, desthiobiotin, and related compounds in bacterial and cellular metabolism are discussed in the recent comprehensive review of Knight (133)

More recently, Tatum (134) demonstrated the synthesis by *Penicillium chrysogenum* of a substance whose biological effects are identical with those of desthiobiotin Pimelic acid, but not cystine, enhanced the synthesis of this substance

Numerous investigations have been devoted to the problem of synthesis of biotin and of other B-complex factors by the intestinal flora These studies have been reviewed in detail by Daft and Sebrell (129) and by Najaar and Barrett (130) It is particularly noteworthy that in the rat a syndrome attributable in part to biotin deficiency can be produced by the inclusion in the diet of one of several sulfonamide compounds which presumably inhibit the synthesis of biotin by the intestinal bacteria (129a, 129b, 129c)

The rôle of biotin and avidin in cancer is under study in several laboratories, and this aspect of the work was recently reviewed by Burk and Winzler (131) Subsequently, Kline, Miller and Rusch (132) reported that the feeding of dried egg white at a 12 per cent level in a purified diet containing suboptimal riboflavin resulted in a reduction in occurrence of hepatomas induced with butter yellow in the rat from a control incidence of from 77 to 82 per cent to from 18 per cent to zero Either heating the egg white or adding biotin to the diet failed to alter the protective effect of the diet containing egg white although the characteristic effects of biotin deficiency were prevented by both procedures This report reopens the problem of the mechanism of the protective effect exerted by egg white

A number of significant studies concerning the effects of biotin upon cellular metabolism have been reported In their early observations Allison, Hoover and Burk (136) appreciated the remarkable stimulus to the respiration of rhizobium afforded by concentrates of the factor which they accordingly termed coenzyme R and which was subsequently shown to be identical with biotin (60, 61) Allison and Hoover later found that the response of rhizobium respiration to coenzyme R depended upon the presence of assimilable nitrogen (137) Burk, Winzler and du Vigneaud (138, 139) have shown that biotin deficient yeasts similarly require ammonia or some other nitrogen source in order to respond to biotin They also found that the effect of biotin upon biotin-deficient yeast is characterized by a prompt rise in fermentation within a few minutes This is followed after an hour by a similar increase in respiration, but the growth response is not apparent for several hours The biotin uptake of deficient yeast was shown to be rapid even in the absence of ammonia, but ammonia was not absorbed in the absence of biotin There was neither destruction nor synthesis of biotin accompanying yeast growth

Burk and Winzler (18) have suggested a possible chemical relationship between the naturally occurring avidin uncombinable form of biotin and the diaminocarboxylic acid derived from biotin which is also avidin uncombinable (19) These authors have postulated that such a substance could function as a coenzyme of $CO_2$ transfer by a reversible conversion to an avidin-combinable compound having a closed urea ring structure similar to biotin

Pilgrim et al (140) reported a marked depression of pyruvate oxidation in liver homogenates taken from both biotin-deficient as well as pantothenic-acid deficient rats Subsequently Summerson et al (141) studied the effects of biotin on the in vitro metabolism of tissues from rats which had been subjected to egg-white injury In liver slices from such animals they observed a slight rise in $O_2$ consumption and R Q , and a very marked increase in the utilization of both lactate and pyruvate The heart and brain preparations from the same animals showed no response to the addition of biotin Assay values given for the liver slices indicated a substantial reduction in biotin content, but no biotin values are given for the heart and brain tissues studied

*General considerations* The studies leading from Boas' original description of egg-white injury (27) to our present knowledge of the subject have constituted a most remarkable research achievement First, the recognition of the egg white effect as a deficiency syndrome led to an appreciation of the existence of a protective dietary factor (27, 51, 52) Persistent study of the nature of this factor indicated its vitaminlike character and something of its chemical and physical properties (40–41, 56–62) The identification of vitamin H with biotin and coenzyme R is one of those rare correlations which bring together segments of information that might otherwise remain unco-ordinated for many years

The next step forward was the demonstration that avidin exerts its characteristic effect by forming an unabsorbable complex with biotin (63–66) Such a mechanism for the production of a toxic effect had not been previously encountered It will undoubtedly serve as a basis for many future investigations in toxicology and nutrition

It should be emphasized that avidin is a normally occurring basic protein sustance found at least in certain specialized tissues of the genital tract in species known to require biotin (7, 74, 76–79, 87, 24) Moreover, it is hardly to be taken as coincidental that egg-yolk is a good source of biotin, while egg albumen has the peculiar property of rendering biotin ineffective (5) Thus, a distinct metabolic relationship between avidin and biotin is suggested and the affinity of this tissue protein for a highly significant metabolite is to be regarded as more than a curious toxic effect

Although our survey has included numerous descriptions of specific biological effects of biotin and of biotin deficiency, these diverse observations give us little indication of the basic metabolic function of biotin Yet its universal distribution in living cells, its profound effects upon bacterial and tissue growth, and its very high level of biological potency per unit weight all suggest a fundamental rôle which is yet to be elucidated

The immediate applicability of our knowledge of biotin and avidin to clinical or technological problems is also obscure Interesting leads have been cited regarding the possible rôle of biotin in resistance to disease (118–120) The clinical usefulness of egg white as a bacteriostatic agent has been reported (135), but such effects may be attributable to lysozyme or other anti-bacterial agents rather than to avidin The suggested relationship between biotin and cancer

(131) is too tenuous to command early clinical consideration Attempts are being made to relate clinical seborrheic dermatitis to the skin changes seen in egg white injury, but no definitive data are as yet available Synthetic biotin analogs which might function as specific anti metabolites have far reaching therapeutic potentialities (20, 20a, 20b, 133)

Despite the considerable body of data already at hand, the avidin biotin problem offers a stimulating challenge for further study

## REFERENCES


(1) DuVigneaud The biological action of the vitamins Chapter V Pp 144–168 University of Chicago Press 1942
(2) Hofmann K. Advances in enzymology Vol 3 289–311 Interscience Publishers N Y 1943
(3) Melville, D B Vitamins and hormones 2 29–66 Academic Press N Y 1944
(4) Wildiers, E Cellule 18: 313, 1901
(5) Kogl, F and B Tonnis Ztschr physiol chem. 242 43, 1936
(6) Eakin, R E , E E Snell and R. J Williams. J Biol Chem 140 535 1941
(7) Woolley, D W and L G Longsworth. J Biol Chem 142 285, 1942
(8) Snell, E E. R E Eakin and R J Williams J Am Chem Soc 62 175, 1940
(9) Hertz R. Proc Soc Exper Biol and Med 52 15 1943
(10) Snell, E E and L D Wright J Biol Chem. 139 675 1941
(11) Wright, C D and H R Skeggs Proc Soc Exper Biol and Med 56: 95 1944
(12) Shull, G M , B L Hutchings and W H Peterson J Biol Chem. 142: 913 1942
(13) Landy, M and D M. Dicken J Lab and Clin Med. 27 1068, 1942
(14) Shull, G M and W H Peterson J Biol Chem 151 201, 1943
(15) Lampen, J O A A Kline and W H Peterson J Biol Chem. (Proc Am Soc Biol Chem.) 140: lxxiv 1941
(16) Cheldelin V H., M A Eppright E E Snell and B M Guirard Univ Texas Publication no 4237 p 26 1942
(17) Lampen J O G P Bahler and W H Peterson J Nutrition 23: 11 1942
(18) Burk, D and R J Winkler. Science 97 57, 1943
(18a) Chu, E J H and R. J Williams J Am Chem Soc 65: 1678 1944
(19) du Vigneaud V K. Dittmer, K Hoffmann and D B Melville Proc Soc Exper Biol and Med 50: 374 1942
(20) Dittmer, K. D B Melville and V du Vigneaud Science 99 203 1944
(20a) Dittmer, K. and V du Vigneaud Science 100 129, 1944
(20b) English J P R C Clapp Q P Cole I F Halverstadt J O Lampen and R O Roblin J Am Chem Soc 67: 295, 1945
(21) Gyorgy P J Biol Chem 119: xliii 1937
(22) Gyorgy P J Biol Chem 131 733 1939
(23) Gyorgy, P R Kuhn and E Lederer. J Biol Chem 131: 745, 1939
(24) Ansbacher, S and M Landy Proc Soc Exper Biol and Med 48: 3 1941
(25) Snell, E E J Biol Chem 154: 313 1944
(26) Stokstad E L. R. J Biol Chem 149: 573 1943
(27) Boas, M A. Biochem J 21 712 1927
(28) Hoagland, C L S M Ward, J E. Smadel and T M Rivers. Proc Soc Exper Biol and Med 45 669 1940
(29) Spence H and E B Schoenbach Proc Soc Exper Biol and Med 49: 415 1942
(30) Williams, R J J Am Chem Soc 66: 896 1944
(30a) Miller, D R. J O Lampen and W H. Peterson J Am Chem Soc 65: 2309 1943
(31) Woods, A M J Taylor, M J Hofer G A Johnson R L Lane and R. J Mc Mahon Univ Texas Publication no 4237 84 1942

(32) Bond, M Biochem J 16 479, 1922
(33) Boas, M A Biochem J 18 422, 1924
(34) Boas-Fixsen, M A Biochem J 25 596, 1931
(35) Friedberger, E Deutsch med Wchnschr 52 1760, 1926
(36) Friedberger, E and S Seidenberg Deutsch med Wchnschr 53 1507, 1927
(37) Scheunert, A and E Wagner Deutch med Wchnschr 53. 1258, 1927
(38) Scheunert, A M, Schieblich and E Wagner Deutsch med Wchnschr 54 689, 1927
(39) Stengvist, F Deutsch med Wchnschr 54 1920, 1928
(40) Parsons, H T and E Kelly Am J Physiol 104 150, 1933
(41) Parsons, H T and E Kelly J Biol Chem 100. 645, 1933
(42) Salmon, W D and J G Goodman J Nutrition 8 1, 1934
(43) Lease, J G, H T Parsons and E Kelly Biochem J 31 433, 1937
(44) Parsons, H T, J G Lease and E Kelly J Biol Chem 100 lxxvii, 1933
(45) Lease, J G and H T Parsons J Biol Chem 105 1, 1934
(46) Parsons, H T and J G Lease J Nutrition 8 57, 1934
(47) Lease, J G and H T Parsons Biochem J 28 2109, 1934
(48) Parsons, H T J Biol Chem 116 685, 1936
(49) Parsons, H T, J G Lease and E Kelly Biochem J 31 424, 1937
(50) Findlay, C M and R O Stern Arch Dis Childhood 4 1, 1929
(51) Gyorgy, P Lehrbuch der Kinderheilkunde, p 562, Berlin, 1933
(52) Gyorgy, P Ztschr f Arztl Fortbild 28 377, 1931
(53) Laurence, W L Science 99 392, 1944
(54) Meyer, K Science 99 391, 1944
(55) Landy, M, D M Dicken, M M Bicking and W R Mitchell Proc Soc Exper Biol and Med 49 441, 1942
(56) Birch, T W and P Gyorgy J Biol Chem 131 761, 1939
(57) Gyorgy, P, D B Melville, D Burk and V duVigneaud Science 91 243, 1940
(58) duVigneaud, V, D B Melville, P Gyorgy and C Rose Science 92 62, 1940
(59) Gyorgy, P, C Rose, K Hofmann, D B Melville and V duVigneaud Science 92 609, 1940
(60) Nilsson, R, G Bjaljve and D Burstrom Naturwissenschaften 27 389, 1939
(61) West, P M and P M Wilson Science 89. 607, 1939
(62) Allison, F E, S R Hoover and D Burk Science 78 217, 1933
(63) Parsons, H T, J Gardner and C T Walliker J Nutrition 19 Supp 19, 1940
(64) Eakin, R E, W A McKinley and R J Williams Science 92 224, 1940
(65) Eakin, R R, E E Snell and R J Williams J Biol Chem 136 801, 1940
(66) Gyorgy, P, C E Rose, R E Eakin, E E Snell and R J Williams Science 93: 477, 1941
(67) Gyorgy, P and S S Rose Science 94 261, 1941
(68) Gyorgy, P, S S Rose and R Tomarelli J Biol Chem 144 169, 1943
(69) Gyorgy, P and S S Rose Proc Soc Exper Biol and Med 53 55, 1943
(70) duVigneaud, V, K Dittmer, K Hofmann and D B Melville Proc Soc Exper Biol and Med 50 374, 1942
(71) Oppel, T W Am J Med Sci 204 869, 1942
(72) Hertz, R Unpublished data
(73) Eakin, R E, E E Snell and R J Williams J Biol Chem 140 535, 1941
(74) Pennington, D, E E Snell and R E Eakin J Am Chem Soc 64 469, 1942
(75) Gyorgy, P and S S Rose Proc Soc Exper Biol and Med 49 294, 1942
(76) Hertz, R and W H Sebrell Science 96 257, 1942
(77) Fraps, R M, R Hertz and W H Sebrell Proc Soc Exper Biol and Med 52 140, 1943
(78) Hertz, R, R M Fraps and W H Sebrell Proc Soc Exper Biol and Med. 52 142, 1943

(79) Hertz, R., R. M Fraps and W H Sebrell. Science 100: 85 1944
(80) Cravens, W W, E. E Sebesta, J G Halpin and E B Hart Proc Soc Exper Biol and Med 50: 101, 1942
(81) Kennedy, C and L. S Palmer Arch Biochem 7: 9, 1945
(82) Snell, E E and Quarles, J J Nutrition 22: 483, 1941
(83) Singher, H. O, C J Kensler, H. C Taylor C P Rhoads and K Unna. J Biol Chem 154: 70 1944
(84) Alderton, G, J C Lewis and H L Fevold Science 101: 151, 1945
(85) Norris, L C and A T Ringrose Science 71: 643, 1930
(86) Hegsted, D M, J J Olson, R. C Mills, C A Elvehjem and E B Hart J Nutrition 21 599, 1940
(87) Hegsted, D M, R. C Mills, G M Briggs, C A Elvehjem and E B Hart J Nutrition 23: 175 1942
(88) Jukes, T H. and F H Bird Proc Soc Exper Biol and Med. 49: 231, 1942
(89) Patrick H, R. V Boucher R A. Dutcher and H C Knandel. Poultry Science 21 476, 1942
(90) Richardson, L. R. A G Hogan and O M Miller Missouri Agric Exper Sta Research Bulletin 343, June 1942
(91) Patrick, H. R. V Boucher R. A. Dutcher and H C Knandel J Nutrition 26 197, 1943
(92) Cooperman, J M., H. A Waisman and C A Elvehjem Proc Soc Exper Biol and Med 52: 250, 1943.
(93) McIntyre, J M., B S Schweigert and C A. Elvehjem. J Nutrition 27: 1 1944
(94) Waisman H A and C A Elvehjem J Nutrition 26 361 1943
(95) Oppel, J Am. J Med Sci 204: 856 1942
(96) Oppel, J Am J Med. Sci 204: 863 1942
(96a) Gardner, J, L. A Neal W H. Peterson and H T Parsons J Am Diet Assn 19: 683 1943
(97) Stokstricker, V P S A Singal, A. P Briggs N M. DeVaughn and H Isbell Science 95: 176 1942
(98) Stokstricker V P S A Singal A P Briggs H M DeVaughn and H. Isbell. J A M A 118 1199 1942
(99) Williams, R. H New England J Med 228: 247, 1943
(100) Fraenkel, G and M Blewett Nature 149 310 1942
(101) Fraenkel, G and M Blewett Nature 150: 177, 1942
(102) Fraenkel G and M Blewett Nature 151: 703 1943
(103) Rosenthal, H and T Reichstein Nature 150: 546 1942
(104) Sullivan, M. and J Nicholls Arch Dermat and Syph 45: 295 1942
(105) Sullivan M., L Kolb and J Nicholls. Bull Johns Hopkins Hosp 70 177 1942
(106) Gyorgy P, M Sullivan and H T Karsner. Proc Soc Exper Biol and Med. 37: 313, 1937
(107) Shaw, J H and P H. Phillips Proc Soc Exper Biol and Med 51 406 1942
(108) McHenry, E. W and G Gavin J Biol Chem 140 lxxxvii 1941
(109) Gavin, G and E W McHenry J Biol Chem 141: 619 1941
(110) Gavin G, J M Patterson and E W McHenry J Biol Chem 148: 275 1943
(110a) MacFarland M L and E. W McHenry J Biol Chem 159: 605, 1945
(110b) Best C H C C Lucas J M Patterson and J H. Ridout Science 103: 12 1946
(111) Fischer, E. Fed Proc 2: 13 1943
(112) Lakers B, J E Thomson and H M Mines. Proc. Soc Exper Biol and Med. 53: 81 1943
(113) Hamilton, H L and H Plotz Proc Soc Exper Biol and Med 50: 133 1942
(114) Burt A S Proc Soc Exper Biol and Med 54: 191 1943
(115) Nielsen E and C A Elvehjem J Biol Chem 144: 405 1942

(116) SMITH, S G Am J Physiol 144 175, 1945
(117) SCHMIDT, J L AND M LANDY Proc Soc Exper Biol and Med 49 82, 1942
(118) TRAGER, W Science 97 206, 1943
(119) TRAGER, W J Exper Med 77 557, 1943
(119a) SEELER, A O, W H OTT AND M E GUNDEL Proc Soc Exper Biol and Med 55 107, 1944
(120) CALDWELL, F E AND P GYORGY Proc Soc Exper Biol and Med 53 116, 1943
(120a) LICHSTEIN, H C, H A WAISMAN, K B MCCALL, C A ELVEHJEM AND P F CLARK Proc Soc Exper Biol and Med 60 279, 1945
(121) EMERSON, G A J Biol Chem 157 127, 1945
(122) OTT, W H J Biol Chem 157 131, 1945
(123) RUBIN, S H, L DREKTER AND E H MOYER Proc Soc Exper Biol and Med 58 352, 1945
(124) DUSCHINSKY, R, L A DOLAN, D FLOWER AND S H RUBIN Arch Biochem 6 480, 1945
(125) HOFMANN, K J Am Chem Soc 67 694, 1945
(126) PILGRIM, F J, A E AXELROD, T WINNICK AND K HOFMANN Science 102 35, 1945
(127) RUBIN, S H, D FLOWER, F ROSEN AND L DREKETER Arch Biochem 8 79, 1945
(128) HOFMANN, K, R H MCCOY, J R FELTON, A E AXELROD AND F J PILGRIM Arch Biochem 7 393, 1945
(128a) HOFMANN, K AND T WINNICK J Biol Chem 160 449, 1945
(129) DAFT, F S AND W H SEBRELL Vitamins and hormones 3 49–72 Academic Press, N Y, 1945
(129) DAFT, F S, L L ASHBURN AND W H SEBRELL Science 96 321, 1942
(129b) MARTIN, G J Proc Soc Exper Biol and Med 51 353, 1942
(129c) NIELSEN, E AND C A ELVEHJEM J Biol Chem 145 713, 1942
(130) NAJAAR, V A AND R BARRETT Vitamins and hormones 3 23–48 Academic Press, N Y, 1945
(131) BURK, D AND R J WINZLER Vitamins and hormones 2 305–352 Academic Press, N Y, 1944
(132) KLINE, B E, J A MILLER AND H P RUSCH Cancer Research 5 641, 1945
(133) KNIGHT, B C J G Vitamins and hormones 3 108–293 Academic Press, N Y, 1945
(134) TATUM, E L J Biol Chem 160 455, 1945
(135) TSYKULENKO, K I Vestnik Oftalmologii 12 511, 1938
(136) ALLISON, F E,, S R HOOVER AND D BURK Science 78 217, 1933
(137) ALLISON, F E AND S R HOOVER J Bact 27 561, 1934
(138) BURK, D, R J WINZLER AND V DU VIGNEAUD J Biol Chem 140 xxi, 1941
(139) WINZLER, R J, D BURK AND V DU VIGNEAUD Arch Biochem 5 25, 1944
(140) PILGRIM, F J, A E AXELROD AND C A ELVEJEHM J Biol Chem 145 237, 1942
(141) SUMMERSON, W H, J H LEE AND C W H PARTRIDGE Science 100 250, 1944

# THE METABOLISM AND PERMEABILITY OF NORMAL SKIN

HERBERT O CALVERY,[1] JOHN H DRAIZE AND EDWIN P LAUG

*Division of Pharmacology Food and Drug Administration, Federal Security Agency, Washington, D C*

While it has been successfully demonstrated that many organs of the body show a relationship between their functional capacity and their metabolism, present information does not permit us to assume a relationship between skin permeability and skin metabolism in man and the higher animals A search of the literature has revealed that in most instances the skin has been regarded as a membrane whose viability was not necessarily concerned with the passage of substances inward Greater stress has been placed on the mechanical and chemical properties of the skin, and it is, therefore, not surprising that the relationship between livingness and permeability has received scant attention

## METABOLISM H O CALVERY

In this section of the review the metabolism of the skin will be considered in its somewhat narrower chemical sense Primary consideration will be given to the more recent literature, part of which has already been evaluated, but from the physiologist's viewpoint (1, 2) Only the skin of man and the higher animals will be considered, and while an attempt will be made to restrict this review to normal skin only, it will be necessary in the case of the vitamins to admit the evidence of deficiency lesions in order to show the relationship of these substances to the normal metabolism of the skin

*Protein* Wilkerson (3) has analyzed human stratum corneum obtained from a case of exfoliative dermatitis, and reported a total N content of 15 09 per cent, calculated on an ash free basis The percentage distribution of nitrogen was as follows Humin N, 2 11, amide N, 3 6, basic N, 30.31, filtrate N, 58 44, ash, 2 6, tyrosine, 5 7, tryptophan, 1 5, cystine, 2.3, histidine, 0 6, lysine, 3 1, and arginine, 10 0 Objection could be made that the distribution of the amino acids in the sample of skin analyzed might deviate from that in skin which had desquamated normally It is believed, however, that the sampling error in attempting a precise separation of stratum corneum in normal skin would be far greater Wilson and Lewis (4) have reported cystine values for normal human skin which are essentially in agreement with those of Wilkerson, although it is not stated how closely the sampling was restricted to stratum corneum

The constitution of keratin, particularly of hair and wool, as representative of this class of proteins, has been extensively studied in recent years by Astbury (5), Huggins (6), and others There is general agreement that they are fibrous structures possessing the phenomena of elasticity and regular intermolecular folding into grid like structures, composed of polypeptide chains with —S—S— (7, 8, 9) and possibly other (6) cross linkages in the polypeptide "grid" It is

to their structure primarily that the resistance of the keratins to penetration and destruction is attributed (10) The protein metabolism of the keratinization process differs from the protein metabolism of the internal cells There is selection of polypeptides, high in cystine and tyrosine, containing large numbers of —S—S— and possibly other bridges, and there is straightening of the coiled polypeptide chains of the cell proteins of the lower layers The amino acids of the keratins are not returned to the circulation by catabolic processes but are lost by exfoliation

The dearth of information concerning protein and amino acid metabolism of the corium is undoubtedly due to lack of the necessary techniques for the study of the specialized processes in the skin Pigmentation, being an obvious, characteristic process of the cells of both the epidermis and derma, has been investigated by both *in vitro* and *in vivo* studies Although tyrosinase has not been demonstrated in mammalian skin, it is generally assumed that tyrosine indirectly, and 3,4-dihydroxy phenylalanine directly, are the precursors of the melanin of mammalian melanoblasts Following *in vitro* investigations of Rothman (11) and others (12–26), the view has been presented that the tyrosine-tyrosinase system may be contributory to the physiology of pigment formation in mammalian skin In addition, factors such as ultraviolet light (11), oxidants, antioxidants (12), adrenal glands (21) and the male and female sex hormones (19) have been shown to affect pigment metabolism Two striking, independent reports by Fessler (27) and by Lewin and Peck (28), should stimulate further investigations These investigators, using spotted guinea pigs, interchanged dark and white skin grafts by transplantation It was found that the white areas were invaded by pigmentation from the surrounded or surrounding dark skin and Fessler reported that this invasion continued for a period of two years For further details on pigment metabolism, particularly from the histological viewpoint, reference should be made to Meirowsky (29)

New techniques for the separation of the epidermis from the corium by means of either heat, chemicals or trypsin have been developed by Baumberger et al (30) and Medawar (31) By the use of chemicals (31) it was possible to separate the epidermis in its entirety, or distal to the basal cell layer, thus affording an opportunity to contrast chemical composition of the different layers The use of heat (30), however, seems far superior to any techniques previously described, since it probably entails minimal alteration of the chemical composition of the cells

*Carbohydrates* The carbohydrate metabolism of normal and pathological skin was reviewed in 1940 by Cornbleet (32) with a good bibliography, therefore, many details need not be repeated here

In table 1 are summarized the average glucose values for normal human and animal skin In the case of human skin, the analyses were made of epidermis plus derma without subcutaneous fat Because of uncertainty of sampling techniques, analytical methods and variations in nutritional state of the skin, some of the values may be subject to revision For example, Pillsbury and Kulchar (40) have found that the presence of glycogen in the skin is a factor

which may produce errors in glucose analysis Cornbleet (32) has found that not only is the sampling site important in judging the glucose content of the skin, but also the layer depth, thus, the average glucose content of a superficial layer was 80.5 mgm per cent but of a deeper layer only 62 1 mgm. per cent As reported by Cornbleet (32) several investigators have studied the ratio of skin glucose to blood glucose and nearly all agree that the glucose level of the skin is directly related to that of the blood, there being a delayed rise and fall in the skin somewhat proportional to the rise and fall in the blood While table 1 shows glucose values higher in skin than in blood, it appears that the truer picture is represented in Cornbleet's analyses Here the blood values range higher than those of the skin in all six species examined The evidence indicates that the glucose of the skin is not bound but is freely diffusible, and its level can

TABLE 1

*The glucose content of skin*

| DATE | AUTHORS | GLUCOSE, MGM./100 GRAMS WET WEIGHT | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Man | Dog | Rabbit | Rat | Mouse | Cat | G pig |
| 1927 | Folin, Trimble and Newman (33) | | 67 | | | | | |
| 1929 | Urbach and Sicher (34) | 47 | 60 | 117 | | 53 | | |
| 1931 | Trimble and Carey (35) | 56 | | | | | | |
| 1932 | Urbach and Rejto (36) | 47 | 84 | 134 | 80 | 53 | | |
| 1933 | Matsumoto (37) | | | 112 | | | | |
| 1933 | Matsumoto (38) | | 91 | 114 | | | | |
| 1934 | Nahara (39) | | | 115 | | | | |
| 1934 | Pillsbury and Kulchar (40) | | | 105 | | | | |
| | | | | 92 | | | | |
| 1934 | Moncorps Bohnstedt and Schmid (41) | | | | | 81 | | |
| 1937 | Urbach, Depisch and Sicher (42) | 61 | 101 | 155 | 104 | 69 | | |
| 1938 | Sellei and Spiera (43) | | | | 139 | | | |
| | | | | | 180 | | | |
| 1940 | Cornbleet (32) | 60-81 | 71 | 97 | 77 | | 78 | 105 |

be influenced by a number of factors For example, insulin, phlorhizin, epinephrine (intracutaneously), cutting the circulation to and from the liver, and topical application of ice, all lower the glucose level, while epinephrine (systemically), histamine (intracutaneously), congestion, local heat, ultraviolet radiation, and chrysarobin, all elevate the glucose level.

Values for the glycogen content of the skin of man, dog and rabbit have been assembled in table 2 Generally speaking, except for the older values of Schondorf, it can be said that the data are more reliable than those for glucose Although the glycogen of the skin may be regarded as less mobile than the glucose, its level does respond to changes in diet (47) or fasting (33) In addition, Cornbleet (32) has listed other factors which more or less influence the level of glycogen in the skin, these are (a) intravenous glucose (slight increase), (b) injection of epinephrine or insulin (increase), (c) stopping blood

flow to and from liver, or intracutaneous epinephrine (decrease) In contrast to the effect on glucose level, factors such as phlorhizin, intracutaneous histamine, topical application of heat or cold, ultraviolet light and chrysarobin produce no effect on the glycogen level in the skin

The lactic acid content of skin has been determined by Pillsbury (48) in animals under a variety of nutritional conditions He found that the lactic acid content of the skin of well-fed animals (range 70 to 138 mgm per 100 grams of skin) was twice that of fasted animals (range 42 to 61 mgm per 100 grams of skin) and that on incubation in dextrose solution there was an increase in lactic acid formation over that of the control Fahrig (45) confirmed these findings

TABLE 2

*The glycogen content of skin*

| DATE | AUTHORS | GLYCOGEN IN MGM. PER 100 GRAMS WET WT (AVG.) | | | COMMENT |
|---|---|---|---|---|---|
| | | Man | Dog | Rabbit | |
| 1903 | Schondorf, by Rothman (44) | 724 | | | |
| 1927 | Fahrig (45) | 157 | | | |
| | | 71 | | | |
| 1927 | Folin, Trimble and Newman (33) | | 17 | | Fasting |
| 1930 | Gualdi and Baldino (46) | | 70 | | |
| 1933 | Matsumoto (37) | | 188 | 86 | |
| | | | (tail) | 82 | |
| 1933 | Matsumoto (38) | | | 87 | |
| 1934 | Nahara (39) | | | 34 | |
| 1937 | Pillsbury and Sternberg (47) | | | 108 | Low carbohydrate diet |
| | | | | 137 | High carbohydrate diet |
| | | | | 75 | High protein diet |
| 1940 | Cornbleet (32) | 76 | 76 | | Avg 33 human subjects, avg 36 dogs |
| 1940 | Cornbleet (32) | 75 | | | Superficial layer } 20 subjects |
| | | 47 | | | Deep layer } |

"Bound carbohydrate", probably glycoproteins (36, 37), glutathione (41), and acetaldehyde (49) have been studied in relation to the intermediary carbohydrate metabolism of skin

*Lipids* The "total lipids" of human "cutaneous epithelium", and cholesterol have been carefully determined in two separate sets of tissues by Eckstein and Wile (50) The first group of eight samples was the exfoliated epithelium from patients having various disorders of the skin and from the soles of cadavers whose skin appeared to be normal These samples were hydrolyzed with alkali and acidified before extraction with organic solvents, therefore, the values represented total lipids less the previously bound glycerol The values for "total lipids" ranged from 2 4 to 9 9 per cent In a second group of four samples, two were thoroughly extracted, the residue hydrolyzed and again extracted This addi-

tional processing added only 4 per cent to the weight of the total lipids found In this second group the total lipid content of the skin ranged from 6 9 to 8.2 per cent, the respective concentrations of some of the constituents of the total lipids were as follows Total cholesterol, 18 to 23 per cent, free cholesterol 16 to 21 per cent, phospholipid, 2 5 to 3.2 per cent

The investigations of others (51–57) have in general confirmed the findings of Eckstein and Wile (50) The study of the skin of the steer by Koppenhoeffer (56) is the most extensive of all chemical analyses of skin reported and should be referred to for details The fresh skin was separated into six approximately horizontal layers including the hair Reference to some of the data which are summarized in table 3 shows that cholesterol and free fatty acids are found chiefly in the epidermal region The evidence clearly indicates that the "basal" epidermal division is the most active metabolically The complex phospholipids cholesterol and its esters, oxidized cholesterol waxes of hydroxy acids with aliphatic alcohols, and saturated hydroxylated free fatty acids derived from seba

TABLE 3

*Distribution of lipids in the skin of the steer*

| | PER CENT OF TOTAL DRY WEIGHT | | | | | |
|---|---|---|---|---|---|---|
| | Epidermal region | | | Transition | Corium region | |
| | Hair | Horn | Basal | | Major | Base |
| Total lipids | 4 60 | 8 72 | 7 28 | 1 95 | 2 44 | 7 15 |
| Cholesterol | 0 67 | 1 01 | 0 97 | 0 22 | 0 06 | 0 08 |
| Lipid P | 0 00 | 0 03 | 0 09 | 0 02 | 0 04 | 0 04 |
| Wax | 0 97 | 2 21 | 2 55 | | | |
| Free fatty acids | 1 24 | 2 09 | 0 40 | 0 21 | 0 06 | 0 15 |
| Acetyl value of total fatty acids | 40 70 | 47 00 | 56 80 | 37 80 | 2 40 | 1 40 |

ceous and epidermal cells constitute a very high percentage of the lipids of the epidermal region

Burtenshaw (57) has contributed important information on the metabolism of the lipids of the skin and their bacteriocidal action He found that oleic acid and other long-chain fatty acids and their soaps were strongly bacteriocidal, particularly at pH below 7 The high antiseptic action of the unsaturated fatty acids was not always related to the degree of unsaturation

*Vitamins* Observations on both man and animals reveal that the skin and its appendages often show striking changes in vitamin deficiencies With the availability of the purified vitamins, it has been found possible to define these relationships more clearly Although the normal metabolism of the skin undoubtedly depends on a proper balance of all the vitamins, it is quite obvious that there is a more direct relationship in the case of some of them than in others Two reviews (58 59) summarize the data available up to 1942

In the normal metabolism of the cells of the epidermis, vitamin A is required to prevent metaplasia and hyperkeratosis of the surface epithelium (60) formed by

the rete malpighii and to prevent atrophy of the epithelial cells of the sebaceous glands with the accompanying accumulation of keratinized cells blocking their ducts

No specific requirements of the cells of the skin for thiamine have been established although there is a general appearance of malnutrition in its absence To maintain normal elasticity, rigidity and color of the skin thiamine is required (61) These are indirect effects probably resulting from the action of thiamine on nerves of the skin As a component of co-carboxylase, thiamine unquestionably must function in the pyruvate metabolism of the cells of the skin

Riboflavin as a component of cellular enzyme systems (the oxidation-reduction mechanism) plays a rôle in skin respiration (62) That it plays a rôle in the capillary dilatation of the skin is evidenced by the pallor which develops in early deficiency and the scarlet redness of some areas which develop later Probably the most characteristic action of riboflavin in skin metabolism is its stimulation of normal hair follicle and hair development In addition, a number of other essential functions have been discovered (a) It prevents atrophy of the sebaceous glands (58), and (b) it is necessary for regular epidermal cell development and cornification processes When a deficiency of riboflavin occurs, it may be frequently manifested by the skin of the face as a greasy, scaly desquamation of the nasolabial folds, bridge of the nose and forehead

Nicotinic acid is another component of the B complex which is specifically required in skin metabolism Without it characteristic lesions of the skin develop These have been amply described in the literature Its deficiency is the primary cause of the lesions of pellagra and black tongue (63–69) The findings of Denton (63, 64) confirmed by Eddy and Dalldörf (65) would indicate the function of nicotinic acid in the skin is to maintain normal water balance and structure of the collagenous fibrils of the basement membrane With this layer in a normal nutritive state, the epidermis, unless otherwise affected as in vitamin A deficiency, remains normal However, when there is failure to maintain the specialized supporting tissues (basement membrane), the epidermis responds with reparative proliferation, dyskeratosis, and atrophy accompanied by vascular engorgement with ectasis and finally cicatrization of the corium The skin of lower animals does not show these changes as clearly as the skin of man

The evidence that pyridoxine is a requirement for human skin metabolism is limited and not conclusive (70) It is, however, required by the rat (71, 72), and probably by the chick, dog, pigeon and pig (58)

There is evidence that pantothenic acid is required by many species for the normal function of cells in general, including those of the skin (58, 59), although its requirement has not been established for man In the skin of animals, it appears that a deficiency of this vitamin affects the epithelial cells of the epidermis, hair follicles and glands The manifestations frequently seen are hyperkeratosis, atrophy, desquamation, alopecia and achromotrichia

Lack of ascorbic acid has been shown to result in petechial hemorrhages, usually located perifollicularly (73), and there is some evidence that this vitamin may also be associated to some extent with pigment formation (19) Biotin pre-

vents avidin dermatitis in the rat (15), while inositol deficiency results in an alopecia and dermatitis in the mouse (75) Petechial hemorrhages and ecchymoses of the skin occur in vitamin K deficiency (76), and the lack of unsaturated fatty acids (77) produces cutaneous swellings, alopecia and dandruff like scaling in the rat.

Vitamin D apparently does not play a direct rôle in the normal metabolism of the cells of the skin, although there is some evidence that it affects the oxygen uptake (78)

*Sweat* The sudoriferous or coil glands are located over the entire surface of the body except the margin of the lips, the glans penis and the inner surface of the prepuce These glands are of two types The apocrine or larger sweat glands are located in the pubic, circumanal, abdominal, mammary and axillary regions These secrete, in addition to an aqueous sweat secretion, an odoriferous substance which is generally considered to produce sex attraction, and hence the apocrine glands are sometimes called accessory sexual glands These glands in the female undergo some change in morphology during the menstrual cycle The most numerous sudoriferous glands are smaller eccrine glands of much more general distribution over the body surface

The product of the sudoriferous glands is both a secretion and an excretion. It is a secretion because the product helps to maintain the skin in a supple condition and preserves tactile sensibility (79) Even the elements which must be considered pure waste products may serve some purpose during their discharge from the body

*Composition of sweat* The acid reaction of the sweat is due to the presence in this secretion of fatty acids, and sodium and potassium acid phosphates McSwiney (80) gives the following average values for the composition of human sweat for 10 normal females and 14 normal males, respectively

| | Mgm./100 cc ♀ | ♂ |
|---|---|---|
| Ammonia N | 4 7 | 6 0 |
| Urea N | 21 44 | 19 23 |
| Amino acid N | 5 0 | 6 5 |
| Glucose | 12 6 | 20 0 |
| Chlorides (as NaCl) | 0 37 | 0 30 |
| Phosphate | 6 14 | 6 57 |

The presence of large amounts of lactic acid in the sweat was not confirmed by McSwiney However, Couraud (81) found in the sweat of healthy subjects total organic acids equivalent to 13 to 45 cc of 0 1 N NaOH/100 cc of the secretion Of this acidity 65 to 85 per cent was due to lactic acid and 10 to 22 per cent to volatile acids Whitehouse (82), in an analysis of the dissolved constituents of human sweat, found wide individual variations in the K, Na and chloride concentrations. The chloride ion concentration was usually greater than 0 15 per cent and occasionally rose to as high as 0.35 per cent The K concentration was between 0.014 and 0 022 per cent The sulphate anion concentration was approximately 0 004 per cent The ratio of organic matter to ash varied with

duration and intensity of sweating and was as low as 1 12 The lactic acid concentration varied from 0 068 to 0 122 per cent and was independent of its concentration in the blood It leads to the conclusion that lactic acid is a normal product of metabolism and a secretion of the sweat gland rather than an excretion from the blood The reaction of the sweat is influenced by the normal acidic reaction of the skin The pH values of sweat collected from the unwashed acidic skin were as low as 4 4

The excretory function of the sweat is illustrated by the presence of many substances similar to those found in the urine These are urea, ammonia, uric acid, creatinine, chlorides, phosphates, sulfates and certain enzymes Concentrations of the above are much lower in sweat than in the urine

Tennent and Silber (83) studied vitamin excretion in male subjects given normal diets and diets supplemented by 250 mgm of ascorbic acid, 50 mgm Ca pentothenate, 10 mgm thiamine chloride and 10 mgm riboflavin daily for one week Thirty minutes before beginning collection of sweat, each subject received in addition 1 gram of ascorbic acid and 50 mgm of the other vitamins Analyses of the sweat showed (a) insignificant amounts of thiamine, (b) no ascorbic acid, (c) 200 micrograms dehydroascorbic acid per hour, (d) 10 micrograms riboflavin per hour, (e) 50 micrograms pantothenic acid per hour (24 micrograms per hour in subjects without supplementation) It is concluded that the loss of vitamins in sweat is too small to cause a vitamin deficiency Sargent, Robinson and Johnson (84) studied the sweat of 11 healthy young men on normal diet produced under various conditions of heat, relative humidity and exercise Fresh sweat was examined for the following substances Ascorbic acid, dehydroascorbic acid, thiamine, diphosphothiamine, riboflavin and nicotinic acid Loss in the sweat of the body's store of water-soluble vitamins was not significant Even under conditions which induce a daily loss of 10 to 15 liters of sweat, excretion of vitamins was considerably greater in the urine than in the sweat

Not in agreement with the foregoing investigators, Bernstein (85) observed that miners exposed to temperatures of 97°F in the Witwatersrand gold mines excreted in the sweat 0 5 to 1 1 mgm of ascorbic acid per 100 cc or about 2 mgm per hour This is offered as a possible explanation for the relative frequency of scurvy among those miners Hardt and Still (86) observed that important quantities of thiamine are lost from the body in human perspiration The loss of ascorbic acid was much lower

### HISTOLOGICAL ASPECTS OF SKIN PERMEABILITY J H DRAIZE

The epidermis of the skin with its imperfections in the continuity of its upper cell layers (1) and its many "pores", (hair follicles and mouths of the ducts of the sudoriferous and sebaceous glands) presents a sieve-like surface It is capable of engulfing considerable material which in a physiological sense has neither penetrated nor been absorbed Material which has been pressed into the skin "holes" and "pores", may appear in the corium if sufficient inunction pressure is applied and if the material is in a sufficiently fine state of division Thus, Milbradt (2) states that carbon particles of small size may, with sufficient massage, be rubbed

into the corium, indicating that the epidermis is not an impervious membrane to such inert materials as carbon particles

This section of the review will limit itself to a consideration of mammalian skin In many of the lower forms of life the skin is the main organ of alimentation respiration and excretion It is also the main sense organ Except when at physiological rest, such skin is freely permeable to water and electrolytes (6) As the organism develops phylogenetically, the skin loses in some degree some of these functions, and in mammalia other organs have assumed the main part of the functions of alimentation, respiration and excretion

It is a characteristic property of warm blooded animals that the horny layer of the skin desquamates constantly and uninterruptedly (1) This sloughing process does not involve an entire layer as in reptiles and amphibia but is a gradual process in which a few cells are shed at a time This gradual desquamation of the outer cells of the horny layer accentuates a primary function of mammalian skin, namely, the prevention of the inward movement of materials which happen to contact the surface of the skin The process of cornification in the epidermis and the epithelial lining of the upper portions of the hair follicles and of the coiled ducts of the sudoriferous glands is further evidence that the skin is constructed to serve as a barrier to the inward transfer of materials The vital processes of mammalian skin, whether these be secretion, excretion or self regeneration, all illustrate one principle, a process intended to exclude to slough and to expel materials rather than to abet their absorption or penetration

The skin in the human is the largest organ It comprises approximately 16 per cent of the total body weight No other organ is equally exposed to as many external influences It serves as the organism's armor in protecting the deeper tissues against trauma and desiccation Histologically the skin is a typically stratified organ, sections of which, by ordinary staining technique, appear to be sharply defined into three layers, the epidermis the corium (cutis or true skin), and the subcutis Although the cutis and subcutis appear to be dissimilar, they are one anatomical entity Embryologically, the skin is derived from two germ layers, the ectoderm and mesoderm The outer stratified portion, the epidermis, is ectodermal in origin This view, however, has not been universally accepted Frieboes (3) attempted to show that the epidermis is derived from the mesoderm the cells of the epidermal layers originating from a transformation and emigration of the endothelial cells of the vascular system of the corium Pincus (4) has reviewed critically the evidence to establish the embryological origin of epidermal cells He concludes that the preponderance of this evidence indicates that the epidermal cells have an origin of their own and are independent of a genetic relationship with the corium

A detailed discussion of the histology of the normal skin is not within the scope of this review and only those structures which by character or architecture affect skin permeability will be discussed For more detailed considerations of the histology of skin structures reference should be made to Pincus (1) and Hoepke (5)

The surface of the skin is covered by a greasy layer consisting mainly of waxes

and free and esterified cholesterol (6) This layer extends inward, so that it also fills the interstices between the cornified cells The water-shedding capacity of the skin, at least for short contact, may be explained on this basis (7) Starken stein's concept (8), however, that the water-impenetrability of the skin is due to this greasy layer, specifically the cholesterol, must be modified (9) Cholesterol itself, but not its esters, is hydrophilic. Stahl (10) has analyzed skin surface fat for cholesterol and finds 14 per cent free and 2 per cent bound, or a ratio of 7 to 1 in the outer surface layer, while in the stratum corneum he finds 10 7 per cent free and 8 9 per cent bound, or a ratio of 1 2 to 1 Thus the outer surface layer of fat appears to have the highest hydrophilic capacity In addi tion to the hydrophilic property of cholesterol, the keratin in the cornified cells tends to take up water and swell It appears, therefore, that the greasy surface layers and the stratum corneum might, according to Leslie-Roberts (11), be con sidered a potential reservoir for holding water soluble substances in intimate con tact with the skin From this favorable depot, it is conceivable that penetration or absorption (in the sense of physiological activity of living cells) may be en-hanced

Since the skin is perforated by gland ducts and hair follicles, the transfer of substances through the skin via these avenues must also be considered The mouths and lumina of these structures are usually occluded by sebum and cell detritus, and even air, which would form for aqueous solutions at least a type of "gas lock" Mechanically forcing materials into the appendages has long been considered the circumstance most favorable to absorption or penetration This is because the lower portions of the lumina and secreting cell portions of the ap-pendages are not lined with dead cornified cells, but with active, living cells It would appear, therefore, that once a substance has penetrated the thin layer of active secreting cells and into a viable portion of the skin it can be truly said that it has passed the barrier of the skin

The epidermis, resting on the basement membrane and corium, consists of five layers of stratified squamous epithelium Beginning with the innermost, and going outwards, the layers are Stratum cylindricum, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum The latter is further dif ferentiated into a flattened-cell layer and a scaly layer The epidermis has no blood supply but depends on the corium for nourishment and fluid exchange Hence the viability of the cells in the various layers as determined by their ap-pearance in microscopic sections is directly proportional to the proximity of these cells to the corium Pincus (1) considers the upper portion of the stratum spino-sum as the level at which cells begin to show deterioration and lessened viability Cells of the progressive layers above this zone become less viable There are no stainable nuclei in the cells of the stratum lucidum and stratum corneum The acidity of the stratum corneum (1, 12) cells is not due to metabolic activity but results from changes due to the death of the cells of these layers

The cells of the innermost or cylindrical layer next to the corium exhibit no abnormality, such as partial flattening and desiccation, as first noted in the upper portions of the prickle cell layer (stratum spinosum) The cells of this latter

layer, in contrast to those of the cylindrical layer, are polyhedral in shape and are further characterized by the presence of numerous cytoplasmic processes (intercellular bridges) or spines which penetrate adjacent cells Characteristic of these epithelial cells are "tonofibrils" consisting of wavy fibrils passing from one cell body to another through the intercellular "bridges" Assuming the basal cell to be in the shape of a cube, one surface of which rests on the basement membrane, Pincus (1) has calculated that the number of "bridges" connecting the five remaining surfaces of each basal cell with contiguous cells is 1092 This interlacing of bridges or spines accounts for a compact layer which is in sharp contrast to the loosely packed cells of the stratum corneum The thickness of the prickle cell layer varies It is thinner over the apices of the dermal papillae, and in those portions of the body where the epidermis is relatively thin there is a sharp change from the prickle cell layer to the flattened, desiccated cells of the horny layer In the areas of thicker skin (e g , palms and soles) there are two well-defined layers external to the stratum spinosum, namely, the stratum granulosum and stratum lucidum The cells of the granular layer are rhomboidal in character and appear to be modified prickle cells As stated above, it is in this layer and in the upper portions of the stratum spinosum that modifications of the normal cells appear The intercellular spaces become narrow and the intercellular bridges become short and indistinct Irregularly shaped granules of keratohyalin are first noted in this layer (1) The origin of these granules has not been established, but as they increase in number and size, the cells exhibit progressive deterioration, the nuclei become pale and disintegrate The keratohyalin represents the first step in the process of cornification in the epidermis. The view (13) has been advanced that cornification is perhaps simply a closer coherence of the tonofibrils due to shrivelling and desiccation of the other cell components. In accordance with this it is pointed out that objective data for the assumption of any other occurrence in keratin formation is wanting The similarity of the normal keratosis to the pathological (parakeratosis) supports the view that the horny layer in these two conditions merely represents the end point of a desiccation process in the cell protoplasm

The stratum lucidum is a thin layer of closely packed angular cells containing a highly refractive cytoplasm The nucleus has disappeared from the majority of the cells and histologically the layer appears as a thin, clear wavy stripe This layer contains eleidin, which is a fibrous protein and an intermediate of the keratohyalin of the granular layer and of the keratin in the horny layer

The stratum granulosum and stratum lucidum comprise the so-called transitional layer of the epidermis It lies between an acidic stratum corneum and a slightly alkaline stratum spinosum The pH of this layer (about 7 0) is close to the iso-electric point of the protein (6) Prolonged immersion in water at pH 7 does not produce swelling of this layer This is in contrast to the horny layer which swells readily under a variety of conditions The impenetrability of the skin to many substances has been attributed to the inherent characteristics of the transitional layer of the epidermis (14)

The most external epidermal layer is the stratum corneum (horny layer) con-

sisting of dead, cornified, flattened cells (1) The margins of these closely packed cells exhibit small irregular spines but the intercellular bridges are absent The first stage in the formation of the cells of the horny layer occurs in the upper portions of the stratum spinosum In this zone the cells begin the process of desiccation, a first step in the process of cornification which is furthered in the immediate overlying layers (stratum granulosum and stratum lucidum) The ultimate stratum corneum cell represents the final stage of desiccation and cornification (6) These cells contain only 10–30 per cent water, whereas the spinosum cells from which they originate contain approximately 70 per cent water The horny layer is hydrophilic and swells readily (15) The horny layer varies in thickness depending, to a degree, on the amount of friction or trauma experienced in the given area It is much thicker in the palms and soles due to much friction and pressure in these areas In the fetus, however, the thickness of the stratum corneum in these areas is also greater than in the other parts of the body, and it would appear that friction and pressure are only partially accountable for the increased thickness The thickness of the horny layer of the epidermis is of insignificant importance in percutaneous absorption In certain pathological skin conditions such as in ichthyosis, where there is a thickening of the horny layer, absorption is not retarded (6) The normal horny layer has been overemphasized as a barrier to absorption and histologically has been characterized as a virtual honeycomb of loosely packed cells (1)

In summary, the epidermis as an integral part of the skin represents a toughened covering membrane which is stratified and so oriented that as the cells of the various strata become farther removed from their food supply (true skin or derma) the structure becomes less viable and more cornified The cells of the most external layer become inert, flattened and hardened elements comparable to shingles on a roof The epidermis is not a perfect "roof" since its upper layers contain many imperfections or "breaks" in its architecture Although these imperfections are present, the effectiveness of the intact skin as a barrier to the absorption of a large variety of compounds has been amply demonstrated (16, 17)

The most characteristic and distinctive components of the upper strata of the epidermis are the keratins (18, 19, 20), the structure of which has been discussed in another section of this review According to analyses of Unna (cited in Pincus (1)), the keratins of the horny layers consist of Keratin A (alkali-insoluble) 13 per cent, Keratin B (alkali-soluble) 10 per cent, with albumoses making up the remainder of the protein portion of the layer It is characteristic of the keratins that they undergo ready swelling in water The imbibition of water with its dissolved substances must play a significant rôle in their permeability of the skin Thus Leslie-Roberts (11) has attempted to explain the initial stages of the mechanism of salicylic acid transfer through the skin by assuming that the acid is first absorbed by the keratins to form a super-saturated solution in the horny layer

*Electrophysical and electrochemical properties of the epidermis* Skin polarization and electro-endosmosis have been studied by Rein (21, 22) Epidermal polarization may be readily demonstrated by passage of an electric current through the intact skin, and is obtained only if the epidermis remains unbroken

It may even be demonstrated on a section of isolated intact epidermis Diehl (23) has used the polarization capacity of the human skin as a measure of its permeability The skin, however, is not a simple membrane Its various layers differ in histological structure and chemical composition Abramson (24) has also pointed out that the skin with its many pores should not be regarded as a simple membrane He concludes that the classical equations for ion and water transport across membranes probably never apply to the human skin Since the stratum corneum is acidic in character and the two lower layers of the rete malpighi are alkaline, the site where neutrality is reached and which accounts for the electrical behavior of the skin are the transitional layers (stratum granulosum and stratum lucidum) These layers may be regarded as the seat of the polarisation current, or the layers which actually account for impermeability of the skin to electrolytes Rein believes the stratum lucidum, particularly, accounts for the electrical behavior of the epidermis Similar conclusions were made by Zeiger (25), who determined the iso-electric range for each layer of the epidermis He found that each layer became more acid the farther it was removed from its blood supply Neutrality is reached at or near the level of the stratum lucidum The transitional layer carried the least charge and has a pH approaching seven and is affected least by foreign agents Pincus (1) regards the stratum lucidum as the layer of the epidermis exhibiting the greatest morphological change with reference to either its preceding or succeeding layers By virtue of its stability it is assumed to be the most impenetrable of the various layers of the epidermis

*The derma* The epidermis rests upon a thin basement membrane which is penetrated by the spines (tonofibrils) of the cells of the stratum germinativum With the advent of better staining techniques, it has been possible to establish (1) that the fibrils or rootlets of the cylindrical layer cells do not penetrate into the corium proper, but are firmly embedded in the basement membrane The very top layer of papillae of the corium is a latticework of fibrillae comprising the basement membrane The derma or corium is the matrix upon which the epidermis and the basement membrane rest It consists of a papillary and a reticular layer which can not be sharply differentiated The papillary layer contains many cone-like elevations or papillae The structure of this layer consists of a fine meshed network of white fibrous and elastic connective tissue In this network of connective tissue are located capillaries and filaments of nerve fibers, —the tactile end organs

The reticular layer comprising the deeper portions of the corium contains bundles of collagenous connective tissue which are coarser and more loosely meshed than those of the papillary layer At the level of the reticular layer are found the larger blood vessels, smaller nerve trunks, sebaceous glands, the more superficial sudoriferous glands and smaller hair follicles

The corium is a highly vascular tissue The papillary layer contains the largest number of the skin capillaries Materials which have penetrated the epidermis and have reached the corium have passed all barriers to absorption except the thin capillary wall Rothman (6) concludes that materials which have reached the corium may be considered as equivalent to having been absorbed

*Subcutis* (*tela subcutanea*) The subcutaneous tissue consists of bands of

areolar connective tissue connecting the deeper layer of the derma with fascia or periosteum This network of connective tissue fibers is filled with lobules of adipose tissue The subcutis contains the largest blood vessels and nerve trunks and the deepest sudoriferous glands, and the coarser hair follicles

The subcutaneous layer, not unlike the corium, is richly supplied with blood vessels, except that the papillary layer of the latter contains many more capillaries Substances which have penetrated to the level of the subcutis are also in favorable position for absorption

*Cutaneous appendages* The cutaneous appendages are the sudoriferous and sebaceous glands, the hair and the nails The sudoriferous glands and their secretions are discussed in another section of this review While the histology of the hair and hair root, papilla and shaft may be important to skin permeability, it is not generally so considered On the other hand, the rôle of the hair follicle is very important, and may not be omitted from a discussion of permeability The hair follicle is an invagination of the skin The walls of the follicle are composed of epidermis and derma One or more sebaceous glands are connected with each hair follicle The epidermis extends inside the hair follicle as far as the opening of the sebaceous gland and consists of the usual spinosum, granulosum and corneum layers Below the opening of the sebaceous gland the hair follicle is lined with the granular and malpighian layers and finally is lined only by the latter Toward the base of the follicle at the level of the blending of the internal and external root sheaths, the epithelium is a mass of undifferentiated epithelial cells The hair shaft does not completely fill the hair follicle, but room is left for the upward movement of the sebum which is discharged directly in the follicle

*Sebaceous glands* The sebaceous glands are branched saccular glands whose ducts open either (a) directly into the hair follicles or (b) onto the free surface of the epidermis The number of glands whose ducts empty into the hair follicles are the most numerous It was long held that the fatty secretions or sebum of the gland was a disintegration of the protoplasm of the glandular epithelium Sebum was thought to be the product of fatty degeneration (Virchow's Fettmetamorphose) and as such represented a non-vital process (1) Melczer and St Deme (26), using a staining technique to study the activity of the gland, conclude that sebum is the product of true glandular activity By their technique they were able to distinguish three different layers, an outer layer which contains mainly unsaturated fatty acids, a middle layer containing higher alcohols, presumed to be formed by the reduction of fatty acids, and an inner layer of neutral fats in the alveolus of the sebaceous gland The individual chemical compounds were not given

*The blood supply of the skin* The epidermis is not supplied with blood vessels This is a factor of importance in the absorption of materials applied topically to intact skin It is not the purpose of this review to describe in detail the blood supply of the skin The reader is referred to Landis (27) and Diasio (28) for the more detailed accounts

The blood vessels of the deeper skin (corium and subcutis) are present in the form of trunks, branches, capillaries, plexuses and anastomoses In given comparable areas the size and number of vessels may exhibit striking variations

The arteries are much smaller in proportion to the veins than ordinarily The main vessel trunks ascend through the subcutis and send branches in all directions. Branches of vessels are also supplied to the fat lobules of the subcutis The large horizontal plexus of vessels at the junction of the deep corium and subcutis sends branches to the sudoriferous glands, hair papillae, vessel walls and the nerves At the level of the mid-cutis the medium size vessels send branches to the sebaceous glands, muscles, vessel walls, nerves and ducts In the upper cutis there is a horizontal plexus of small vessels from which extend branches to the papillary bodies, muscles and ducts In the skin, capillaries are found almost exclusively in the upper cutis Since the epidermis is not furnished with a blood supply, the relatively rich capillary bed in the upper corium appears to be partial compensation to insure the best possible fluid and metabolic exchange Equally important from our standpoint, however, is the presence of a rich vascular bed to receive materials passing the epidermal barrier and which are in position for systemic absorption The sudoriferous and sebaceous glands, the hair papillae and the lobules of adipose tissue receive their blood supply through a capillary network derived from recurrent branches of various vascular plexuses

Each papilla is normally provided with a central loop, the arterial branch of which is generally narrow, while the tip of the loop and the venous branch are often much wider The length of the loop varies and the number of capillaries in the papilla is small compared with those in muscle The average number of capillaries in the skin of the hands, face, forearm and circumoral skin is 16 to 65 per square millimeter (29) This is a relatively small number compared to muscle where it is estimated that the number of capillaries varies from 1,000 to 2,700 per square millimeter (30) The papillary capillaries supply the germinative layer of the epidermis with substances necessary for its continuous growth

The arterial capillaries end in multiple anatomoses with the venous capillaries The latter combine to form venules which return to a subpapillary plexus of small veins situated just below the papilla From this plexus of veins small trunks descend beside the arteries and empty into another plexus in the deep cutis This latter plexus also receives veins emanating from the sudoriferous glands and from the fat lobules Veins of the deep cutis finally drain into the subcutis plexus which terminate in the large subcutaneous veins

The skin is richly supplied with lymphatic vessels The presence of lymph canals in the epidermis has been demonstrated by Melczer (31) by the use of urea solution and subsequent precipitation and fixation by mercury salts. Dense flat meshworks of lymphatic capillaries originate in the papillary layer of the corium either as networks or as blind outgrowths The lymphatic capillary networks are deeper than the blood vessels. The more superficial networks of lymphatic vessels send branches to other plexuses at the boundary of the derma and subcutis. From these deeper networks originate large lymphatic vessels which follow the blood vessels Lymphatic vessels are not supplied to the cutaneous appendages (hair and glands of the skin) This is significant when it is considered that many substances are absorbed principally through the skin appendages

*The innervation of the skin* Woolard et al (32) and a recent review of

cutaneous sensation by Walshe (33) treat the subject of skin innervation and sensation in detail The skin is richly supplied with large nerve trunks, both sympathetic and cerebrospinal Nerve branches supply directly the larger blood vessels, hair follicles, the sebaceous and sudoriferous glands, and the tactile corpuscles

The reticular layer of the corium contains plexuses of nerve fibers, while more closely meshed plexuses of finer nerve bundles innervate the papillary layer Smaller fibrils are distributed to the smaller blood vessels and to the papillae where many end in tactile corpuscles Certain fibrils penetrate into the epidermis, terminating as naked fibrils or on tactile cells

Small branches form a network of fibrils in the root sheath of the hair follicles Pilomotor nerves are also distributed to the arrectores pilorum muscles In the sudoriferous glands fine plexuses send axis cylinders through the basement membrane, terminating in the secreting cells

The corium and subcutis are richly supplied with blood vessels and nerves Substances which have penetrated to this level may, by virtue of their irritant effect on nerve and tissue, cause significant local vasomotor disturbances These may be either a vasoconstriction or a vasodilatation, and in either case such a change in the condition of the vascular bed affects both the degree and rate of fluid exchange at the site Calvery et al (16) have shown that many substances topically applied elicit an inflammatory reaction in cutaneous structures Such reactions cause changes in blood vessel permeability resulting in edema formation The edematous tissues are no longer capable of maintaining normal fluid exchange, and absorption in such cases becomes retarded On the other hand, substances which do not affect vessel permeability but produce vasodilatation produce local conditions in tissues favorable to increased absorption

PERMEABILITY E P LAUG

At the outset, it should be remarked that the term "permeability" probably is not entirely satisfactory, because it may connote to some a process of purely physical transfer Thus, the membrane in this case, the skin, could be considered acting as a passive filter, its livingness being of importance to permeability only in the maintenance of electric properties, interstitial spaces, condition of the cell wall, etc , but that no secretory activity of cells would be involved, whereby a substance would be taken up, concentrated and pushed inward For this latter property of active work on the part of the skin cells in transferring substances inward, the literature offers little or no clearcut evidence It is true that Stejskal (34) has taken the position that if certain cells of the skin show active secretory properties in transferring substances from the inside to the outside, sweat, sebum, etc , why should not these same or similarly constituted cells be able to secrete inward? The point is hardly well taken

The term absorption, which certainly conveys the sense of cellular activity, is frequently (inadvertently ?) used Here it may be assumed that the investigator has in mind a purely physical process of imbibition, whereby, for example, water may be taken up by a piece of filter paper, dried gelatin or keratin The

term penetration is also used but in contrast to absorption and permeability, both of which put the emphasis on the membrane, penetration can be thought of as emphasizing the substance and those of its intrinsic properties, which make it able to enter into or overcome a barrier It may make little difference whether the more or less resistant membrane or barrier is a living or dead membrane

The literature on the subject of skin permeability has singularly failed in making clear-cut distinctions between terms, perhaps this is in part traceable to the fact that many investigators have been more concerned with what substances went through the skin rather than why or how they went through the skin

Generally speaking, the freedom with which liquids and gases may pass across the skin both inward and outward is a property which increases as we descend the phylogenetic series In the case of the frog and other amphibious or aquatic forms, this property is quite marked and has been the subject of extensive studies which would form the basis for a review in itself In man and the higher forms it appears that this property of two-way passage has become restricted, so that while retaining its excretory functions, the skin may have lost not only most of its passive (permeability) but also its active (absorptivity) capacity of transfer of substances inward

*Historical* For a resumé of the historical literature on the subject of skin permeability, reference should be made to Rothman (6) Broadly sketched, the subject may be divided into three periods (a) The period antedating 1877 and covering over a hundred years, during which much of the evidence was accepted as indicating that a large number of substances, chiefly gases and volatile substances, penetrated the skin freely (b) The period extending from 1877 until about 1900, during which Fleischer's (35) school of thought held sway Fleischer and his collaborators concluded from their own experiments, and a critical appraisal of the older observations, that the skin of man and the higher animals was absolutely impermeable to all substances (c) The period from 1900 to the present The first years saw the decline of the absolute impermeability idea chiefly under the impetus of Schwenkenbecher's work (36) By the use of more refined analytical techniques, it is now possible to determine that many substances are more or less able to pass through the skin The consensus, however, is cautious, and does not recognize the more extreme position of Stejskal (34), who claims from an uncritical appraisal of the literature that practically all substances are able to pass through the skin Generally speaking, it appears as though the dermatologists were still trying to disprove the idea of skin impenetrability and there continue to be literally hundreds of instances reported of percutaneous passage

*Factors concerned with skin permeability* According to Perutz (7) there are three fundamental factors (a) The substance which penetrates, permeates or is absorbed (b) The vehicle which affects transfer of the substance (c) The skin itself Since the last is the subject of a separate section of this review it will not be considered in detail here It is the purpose of this review to discuss the various groups of substances that have been shown to penetrate or not to penetrate the skin, and to evaluate where possible the function of

the vehicle The problem might be relatively simple were it not for the fact that interactions between the skin, the vehicle and the substance may and frequently do occur Failure to recognize this is responsible in part for an extremely large background of confused and contradictory literature in which the penetration of salicylates and iodine stand as notable examples

*Methods* The most commonly used method to determine whether a substance has passed through the skin is its chemical detection in the urine Other, but less extensive analyses, have been made of tissue and fluids of the body, and in the case of volatile substances, expired air Obviously, the chemical method must be sufficiently delicate to detect very small quantities of a substance gaining access to the body via the skin Due to the slowness with which some substances may pass through the skin, or be released from possible subcutaneous, or other depots, the final dilution in a 24-hour sample of urine may be so great as to escape chemical detection This fact has not been sufficiently emphasized by some investigators when reporting negative results Recently (37) a successful attempt was made to measure skin permeability to mercurial ointments by utilizing the capacity of the kidney to store the mercury which had entered the blood percutaneously Under the conditions of the test, the kidney contained on a per gram basis from 50 to 100 times as much mercury as the rest of the body—or even the urine One of the limitations of the chemical method lies in the interpretation of results obtained on studies of skin permeability of common body constituents, such as glucose, sodium chloride, carbon dioxide, etc If the "background" is too large or too variable, it becomes impossible to interpret the results It is for this reason that the knowledge of skin permeability has lagged with respect to the common constituents but has made considerable progress with respect to substances foreign to the body

In addition to the chemical method, local and systemic pharmacological and toxicological signs and symptoms have been relied upon to demonstrate the passage of an active penetrant through the skin Even where the delicacy of the biological reaction is on a par with the chemical, rejection of negative results may be made on some of the same grounds as stated above In many cases, of course, the systemic response requires a certain minimum amount of material to be present at one time, a situation which may not be fulfilled, due either to the slowness with which the substance passes through the skin, or the rapidity with which it is destroyed within the organism

It should be quite obvious that, in the conduct of skin permeability studies, meticulous care must be taken to insure that the avenue of entrance into the organism is the skin, and the skin only Many conflicting data have arisen through failure to observe such a simple requirement [2]

Generally speaking, knowledge of skin permeability to a variety of substances

[2] The famous controversy over the clinical use of mercury inunction Some asserted that the efficaciousness came from inhalation of mercury volatilized from the skin, others, from passage of mercury through the skin Under the proper conditions the latter avenue is the more important Oral contamination is sometimes not too easy to circumvent, particularly in animals

has been widely extended by application of the principles just described Unfortunately, by their very nature the methods have contributed little directly to our understanding of the mechanism involved

In comparison with other methods, the histological and histochemical have probably yielded the most information on the manner in which penetration of the skin occurs At best, these methods have only been able to locate the penetrating material within the epidermal skin structures, and the probable avenues of approach to the basal layers which are considered the ultimate barriers With some exceptions (38, 39), which will be discussed later, no clear-cut demonstration of how a substance passes the basement layer of living cells (rete malpighii) has ever been made The substances most frequently studied by this method have been fats and oils. Fat soluble dyes have been added in order to follow the course of the penetration, the assumption being made that the dye and its vehicle move together This is probably not entirely justified for the skin contains its own fats for which the dyes might have greater affinity In other techniques, fats are injected without dyes and the skin stained after sectioning By comparison with control sections, the distribution of the injected fat is followed Here, too, extreme caution in the interpretation of the results must be observed particularly since little is known concerning the effect of various mechanical and chemical manipulation on the distribution of the natural fats of the skin By contrast with the fat distribution studies, it would appear that the histochemical tracing of substances foreign to the skin, such as sulfur (40) or heavy metals such as lead (41) and mercury (42, 43), within the epidermal structures might be more trustworthy

Where the chemical method for determining substances within the organism after passage through the skin has been limited, the method of "analysis by difference" has proved useful Essentially it consists in applying a carefully measured amount or concentration of the substance to the skin, and after a suitable time, determining how much still remains As used by Hediger (44) and perfected by Burgi (45), much valuable information has been obtained In practice, a bell-shaped glass vessel is cemented to the skin, and filled with the substance to be studied If the substance is a gas, pressure or volume changes may be recorded, if the substance is in solution, samples may be removed at intervals and changes in concentration determined Wild has applied the principle to studying the penetration of grease bases (46) and mercury ointments (47) He rubbed weighed amounts of the preparations into the skin of the forearm, and at intervals removed the excess material to weigh and analyze The results on repeated trials were remarkably consistent

The chief use of the "analysis by difference" method has been in the study of constituents common to the body, such as NaCl, KCl, $CaCl_2$, $CO_2$, $O_2$, etc If the "remainder analyses" are very small, however, the results may well be regarded with suspicion since the weakness of the method consists in assuming that when a substance disappears from the skin surface, it has passed through the skin and entered the organism It is not impossible that considerable amounts of material alleged to have "penetrated" may be lodged in the skin ap-

pendages Here it may be judged just as much "outside" the body as any unabsorbed materials within the intestine On the other hand, if the "remainder analyses" are large, as, for example, in the case of $CO_2$ and $O_2$, there cannot be much doubt that the skin has been completely permeated

*Lipoid solubility* Probably the most important factor governing permeability of the skin is lipoid solubility The subject has been discussed at length by a number of authors (8, 48, 49, 36) The presence of lipoid material, either surrounding the individual cell, or lodged as amorphous material between cells, or within the openings of the appendages of the skin determines to a certain extent what shall pass (10) Since the milieu consists of water also, complete insolubility of a substance either in water or in lipoid would be a property which could hinder or prevent penetration (6, 50, 51) Thus the distribution coefficient Lipoid Solubility/Water Solubility of a substance is probably an extremely important factor In the case of an electrolyte we have water solubility and lipoid insolubility In the case of a fat we may have the reverse In either case the evidence for passage through the skin is poor Theoretically, therefore, we may expect that a large class of organic solvents, such as alcohol, aldehydes or ketones, or those having groupings that confer both water and lipoid solubility, should penetrate the skin with ease

Generally speaking, as the tendency for ionization of a compound decreases, its lipoid solubility increases but, of course, there are important exceptions, such as the sugars and the amino acids These substances are lipoid insoluble (50), and there is not much evidence that they can pass through the skin Among the inorganic substances we find that the heavy metals are definitely lipoid soluble and form poorly ionizing compounds There is also evidence that they can pass through the skin, a good example is mercury which will be discussed later

An example where change in ionizing and lipoid soluble properties modifies the ease of penetration through the skin is salicylate As the free acid, this substance is lipoid soluble, slightly ionized and penetrates the skin with ease, as the sodium salt, it loses its lipoid solubility, ionizes freely, but for all practical purposes no longer penetrates the skin For a review of the older literature, reference should be made to Rothman (6) A similar example is boric acid and its sodium salt (Kahlenberg, 52)

*Vehicle* Two general types may be recognized, (a) Ointments or salves, consisting of various kinds of fats, fatty acids, waxes and lipoid substances such as cholesterol and lecithin, (b) organic solvents, such as alcohol, chloroform, etc Generally speaking it may be said that the vehicles exert a marked effect on the penetration of substances through the skin, although some investigators feel that the nature of the penetrating substance rather than the vehicle may be the determining factor Bliss (53) epitomizing the latter viewpoint writes "The properties and powers of the drug itself rather than the ointment vehicle are the major determining factors in absorption from the skin " This statement, however, is based on tests with a restricted number of substances only Macht (54), after studying a number of alkaloids, concluded that ointment vehicles did little to improve but may even hinder their penetration He also found that iodine was

absorbed better from a tincture than from an ointment Amrhein (55), in reporting cutaneous penetration of vitamin D, noted that the ointment vehicle had little or no influence on penetration

Against the foregoing statements there is a wealth of evidence which supports the contention that the vehicle definitely influences penetration Thus for salicylic acid, which has been so extensively studied, differences between wool fat, vaseline, lard, etc , were shown by earlier investigators (51, 56), and more recently by Matschak (48), Leslie-Roberts (11) and Moncorps (57), the last investigator finding as high as 40 fold differences in penetration between certain types of ointment bases Effects on the penetration of sulfur (40), mercury (43, 61) and KI have been noted In the case of the last, which penetrates very poorly or not at all from aqueous solution, Canals and Gidon (62) were able to show measurable penetration when this compound was incorporated in vaseline or fat For additional earlier literature on the effect of ointments on KI penetration, Rothman (6) should be consulted

There is no good evidence that the ointment base acts as a vehicle to carry the substances through the skin (63) This is indicated by the poor penetration of the fatty constituents themselves (See section on fats ) Recently, Czetsch Lindenwald (64) reinvestigated certain ointment bases for penetration through the skin His results were completely negative for cholesterol, vaseline and saponifiable components of the ointments After application of a salve made of hydrated oil containing 33 6 mgm. per cent of deuterium, no deuterium oxide could be demonstrated in the products of combustion of the body fats

The manner in which the ointment vehicles help to increase penetration is largely mechanical (6) Being of greasy consistency they can easily be spread over the surface of the skin and are able to bring the penetrating substance into more immediate contact with the skin As a matter of fact, ointment bases which are not supple, but stiff and sticky or which tend to dry out and flake off, usually support but little penetration (61) According to Strakosch (65) there is no good evidence that penetration directly through the stratum corneum is enhanced by the ointment, rather the ointment tends to be forced into the appendages, specifically the hair follicles and the pilosebaceous glands, from where penetration or absorption can then occur Air which is trapped within these appendages, and which might serve as an effective barrier to the penetration of aqueous solutions (51, 66), can also be displaced, particularly when the inunction is accompanied by a certain amount of pressure Ointments applied to the skin may also interfere with the normal excretion of water as perspiration and insensible water transfer It has been shown that the application of a film of vaseline reduces the insensible perspiration of skin by 40 per cent (67) Water which continues to be given off beneath this greasy covering, since it cannot escape, is imbibed by the cornified epithelial cells (68) which tend to swell and become "water logged" In this condition it is possible for a local concentration of a water soluble penetrating substance to occur in the outer cornified layers (11) Physical and chemical interaction between the ointment vehicle and the incorporated substance may result in either hindrance or enhancement of penetration Aside

from its ability to get the penetrating substance into more intimate contact with the skin, the vehicle must also be able to part with the substance and not "hold" it, otherwise penetration may actually be obstructed This is a situation which may account for some of the apparent differences between ointments, and has received consideration by a number of investigators (48, 68, 69) and (70) There is also evidence that chemical reactions between vehicle and incorporated substances lead to modification of penetration, for example, ester formation of salicylic acid with cholesterol (11, 48), oxidation of iodine in KI by peroxides formed from the reaction of water with fats (71, 72), and reaction of mercury with fatty acids (43,60)

Organic solvent vehicles enhance percutaneous penetration considerably, but it is somewhat difficult, in attempting to explain the mechanism, to separate the lipoid dissolving action from the vehicle action According to the older literature (73, 74) pretreatment of the skin with chloroform, ether or alcohol, enhanced the penetration of water soluble alkaloid salts This would make it appear, according to Winternitz (73), that the wettability of the skin surface and the walls of the hair shafts and sebaceous glands had been increased by the removal of water repelling lipoid materials, a condition favorable for the penetration of water soluble substances These same investigators also found excellent penetration when the substance was applied to the skin as a chloroform, ether or alcohol solution In the modern literature there is also good precedent for using lipoid solvent vehicles, as witnessed by the successful application of sex hormones in alcoholic solution (see section on sex hormones) Alcohol, ether and chloroform (45, 74, 75, 36) are in themselves able to penetrate the skin Consequently, it may be assumed that they can act as transporting vehicles for other substances, but since chloroform, ether and alcohol are also skin irritants (59, 75), there may be considerable doubt how "normal" skin is under such conditions

*Electric current* The use of the electric current as a "vehicle" for the transport of substances through the skin has been amply considered in the older literature which has been reviewed by Rothman (6), Leduc (88), and Frankenhauser (89) It is not proposed to discuss the clinical aspects of this subject except insofar as they may throw light on the mechanism In general, it can be asserted that by the application of a direct current, it becomes possible to move a large number of substances which would otherwise penetrate poorly through the skin There is still some confusion in the use of terms to designate the process, Rothman (63) prefers electrophoresis since this is a general term which includes cataphoresis, iontophoresis and electro-endosmosis

The method consists in placing gauze or cotton saturated with a solution of the substance on an area of the skin, covering this with the active electrode of zinc or aluminum and pressing down firmly The circuit through the skin is then completed by attaching to some other skin area an "inactive" or neutral electrode moistened with water A current density of about 1 milliampere per $(cm)^2$ area is allowed to flow for a few minutes up to one-half hour If larger areas of skin are to be exposed, the active electrode is made the fluid into which an area or segment of the body dips

A noteworthy contribution to the understanding of the process of electric transfer of substances through the skin has been made by Rein (22, 76, 77, 78 79, 80) who extended the earlier work of Ehrman (81) in using dyes to visualize the avenues of entrance In agreement with this work, Rein (79) finds that the transport occurs almost exclusively through the hair shafts and sebaceous glands Thus with methylene blue which gives positively charged dye ions in solution, a characteristic pore pattern was visualized in the skin beneath the anode The mouths of the hair shafts and sebaceous glands were seen to be deeply colored Histological examination revealed that the dye had penetrated into the fundi of these structures and thence out through their walls and into the surrounding rete malpighii, and thence into the corium Recently Abramson and Engle (82) have produced pore patterns in human skin which show that electric transport seems to favor the sweat glands As for the third possible avenue, namely, the epidermis itself, the evidence for direct passage is poor In those cases where there was a diffuse coloration of the epidermis which did not seem to originate from any channel structures such as the hair shafts or sebaceous glands, Rein (79) showed that there was evidence of skin cell injury characterized by vacuolization and loss of nuclear staining

The earlier literature indicated that the process of transfer was by iontophoresis, i.e , the movement of ionized basic and acidic elements This is very clearly shown in the classical experiment of Leduc (83) in which strychnine sulfate was applied to two rabbits electrically connected by a salt bridge Here the animal whose skin was in contact with an anode moistened with the alkaloid salt died within a few minutes, while the other animal in contact with the cathode similarly moistened showed no toxic symptoms Similar toxic results could be obtained with KCN in the animal connected to the cathode lead (84) Rein (22, 76), however, showed that while iontophoresis was involved, by far the most important mechanism was endosmosis, i e the transfer of water together with the ions According to Rein (80) the skin (specifically the stratum lucidum) is constituted as a negatively charged membrane Water, therefore, moves inward across the barrier from an anode placed in contact with the skin This is electro-endosmosis, and disturbance of local fluid balance is shown by the shrinkage of the skin beneath the anode Positive ions, of course, also move in this field, and in the same direction (methylene blue+, atropine+), thus the over all effect is an enhancement of penetration However, when an attempt is made to move negative ions, such as eosin, into the skin from the cathode, the electro-osmotic stream is outward, and opposes the inward ion movement, hence, there is practically no penetration (79) Superficial examination shows that in certain areas under the cathode there is a diffuse coloration, but closer histological inspection shows that this is due to injury to the cells (79) Certain spots appear as though a "break through of the stratum lucidum barrier had occurred" Abramson and Gorin (66) claim to have transferred successfully negative dye ions into the skin from the cathode, but in view of the foregoing this may be regarded as due to injury (79, 85, 86) Salt solutions, polyvalent positive ions and acid tend to reduce the negative charge of the skin (80) and practically abolish the penetration

of methylene blue (79) or histamine (87), conversely, such treatment tends to increase the tendency for the transfer from the cathode of negatively charged ions Electro-endosomotic transfer, according to Rein (78), is favored by anything which reduces the specific conductance of the solution such as addition of alcohol or sugar, or the reduction in concentration of ions In addition to electro-osmosis and iontophoresis as the chief mechanism for electric transfer of materials through the skin, Abramson and Gorin (90) have advanced evidence for what they believe to be a third, namely, diffusion over small distances under the influence of the electric current In special cases where electro-osmosis and iontophoresis balance one another, this last mechanism is supposed to operate These investigators cite histamine as an example With this substance there is retardation of diffusion in the skin except under a potential gradient, following its introduction into the skin by electric means it may remain in situ for a week, unless moved by electric means

*Functional modification of the skin* It can be said without reservation that a great deal of confusion and contradictory statements regarding skin permeability have arisen in the literature through failure to recognize or properly evaluate the effects of functional modifications of the skin Some of these changes involve frank injury and are, of course, fairly easily recognized It is generally agreed that agents causing inflammation, vesication, keratolysis or mechanical lesions, gross or microscopic, should not be included in any consideration of "normal permeability" Some of the chemical agents in such a category are mustard oil, croton oil, phenol, polysulfides, alkalies, chloroform, turpentine, saponins and chrysarobin On the other hand, it may also be said that there are few substances which when placed in contact with the skin do not alter its properties or functions Even covering the skin (2) with various types of bandages, air and water tight coverings, or a layer of vaseline may alter the normal function of the skin in preventing water loss from the surface While such a procedure may not cause injury, in the sense described above, it certainly does cause marked physical changes in the surface keratinous layers of the skin These layers imbibe the water which is held by the covering, and tend to swell In this condition a more favorable surface for penetration of some substances results (61) It becomes difficult to draw the line between altered function and injury, or between physiological stimulation and mild irritation It is conceivable that milder forms of "injury", or what might be termed temporary modification of structure or function of the skin probably change its permeability (2) to a number of substances As examples, there may be cited (a) mild keratolysis, (b) hyperemia, (c) removal of skin constituents such as fats or cholesterol by precipitation or solution, (d) mild irritation or stimulation of epidermal cells

(a) *Mild keratolysis* Unna (68) inclines to the view that the outermost keratinous layer of cells is a partial protection against the penetration of many substances He assumes that phenol, salicylic acid, caustics, etc increase the permeability of this layer by their keratolytic action Under certain conditions where salicylic acid is applied to the skin, Moncorps (57) has been able to correlate the passage of this acid through the skin with the degree of keratolysis of the

surface layers Kionka (91) also assumes that the penetration of salicylic acid is associated with its keratolytic action Milbradt (2) studied the keratolytic action of salicylic acid, pyrogallic acid, resorcinol and $\beta$ naphthol on the penetration of dyes. Following their reaction with the skin a marked increase in the penetration of all dyes was noted, in particular trypan blue Penetration was observed to be most active around the hair shafts and extended outward therefrom into the middle layers of the corium Rothman (92) denies that mild keratolysis can increase skin permeability, on the grounds that reduction in the thickness of the dead cornified layer can have no effect on the over all permeability of the living cells beneath Nevertheless, it is difficult to conceive that keratolytic action should be limited sharply to the surface layers of cornified cells It would seem that the question should remain undecided until more histological studies can be brought to bear on this point

(b) *Hyperemia* It has been assumed generally that simple hyperemia enhances the penetration or absorption of substances through the skin (93, 51) Specifically Kramer and Sarre (94) have observed a 4 to 5 fold increase in the uptake of $CO_2$ by the hyperemic skin as compared with the normal Rothman (63), however, takes the position that hyperemia cannot in itself increase absorption or penetration because the "barrier", the epidermis, lies above the capillary bed A substance must first have passed the "bloodless barrier" before it can be removed by the increased circulation of blood in the corium It must be remembered, however that whatever produces hyperemia may also modify the permeability of the rete cells

(c) *Stimulation or irritation* In addition to causing an expansion of the capillary bed, mechanical, chemical and physical stimulation may produce increased cell permeability In the skin this is reflected by a lowering of the electric resistance of the skin, as for example, after the passage of a galvanic current (85, 95) or exposure to ultraviolet light (96) although in the latter case (97) the phenomenon occurs only after erythema has been induced By the use of dyes, histological evidence of increased cellular activity has also been noted after ultra violet light (98), and specifically nearly a doubling of permeability of skin cells to ammonia (99) Frank damage characterized by vacuolization following penetration of eosin from the cathode (79) has been observed By chemical stimulation, for example, it has been possible to advance salicylic acid much more rapidly through the skin by means of capsicum (91), potassium iodide, and by pretreatment of the skin with pine needle extract (71)

Milbradt (2), in experiments for which unfortunately no protocols are given, has studied the penetration of trypan blue and other dyes into skin pretreated with croton oil and other irritants In every case he observed histological evidence of tissue irritation, such as increased mitosis, expansion of the capillary bed, and increase in the space between cells (Gewebsauflockerung) In every case also, there was markedly deeper penetration of dye than in the untreated skin Peculiarly enough, the coloration seemed to transcend the epidermis very evenly, only occasionally were the skin appendages more deeply colored than the rest of the skin structures

(d) *Removal of skin constituents* Much could be said on this score Since the location of fats and lipoid bodies on the surface and within the interstitial spaces of the outer layers of the epidermis may constitute a natural barrier to the passage of many substances into the skin, their removal creates a decided modification of the epidermis Thus, aside from their primary irritating qualities, ether, chloroform, phenol and a host of other organic solvents, by their solution and removal of lipids and cholesterol, are frequently considered for their properties of enhancing penetration Even alcohol (2) in 50 per cent dilution has this effect, although at higher concentrations, permeability of the skin is decreased due to the precipitation of tissue proteins The classical experiment of Winternitz (73) is frequently cited When the skin of a rabbit was treated with strychnine, systemic reaction followed very slowly, when the skin was pretreated with chloroform, or the alkaloid dissolved therein, systemic reaction followed very swiftly Similarly, pretreatment of the skin with petroleum ether (101) enabled insulin to penetrate and lower the blood sugar It is extremely doubtful whether any of the above lipoid solvents can be included within the considerations of "normal" skin permeability Mulbradt (2, 102) has found that saponin enhanced the penetration of dyes, epinephrine, iodine, salicylic acid, cinchophen, etc In the case of trypan blue, saponin-treated skin was much more deeply penetrated than untreated skin The coloring showed in the middle layers of the corium, particularly around hair shafts Besides its irritant action, which is noticeable above concentrations of 5 per cent, the chief action of this glucoside is the precipitation of cholesterol Saponin, therefore, seriously modified the normal lipoid structure of the skin

*Water* There is still much difference of opinion as to whether water can penetrate the skin from the outside to the inside In reviewing the older literature, Schwenkenbecher (36) arrived at the conclusion that the skin of man and the higher animals offered a nearly complete barrier to the entrance of water A frequently cited, but old observation of Fleischer (35) offers evidence for the complete impenetrability to water This investigator immersed the whole arm in a plethysmograph filled with water and carefully sealed to prevent leakage By means of a manometer connected to the plethysomograph, changes in the volume of water could be recorded After making allowances for certain experimental errors, Fleischer concluded that in a period of one hour no water passed inward through the skin Stejskal (34) states that the ability of water to penetrate inward through the skin can no longer be doubted and rejects the older experiments of Fleischer His evidence for the penetration of water, however, is based largely on experiments of dubious value He cites, for example, an early attempt (103) to measure changes in the total water content of the body by weighing before and after immersion in a bath, a technique involving gross errors Even the recent carefully conducted experiments of this type by Whitehouse et al (104), which purport to show that water passes inward through the human skin under certain conditions, are not above suspicion

It thus becomes apparent that the experimental evidence for or against the passage inward of water through the skin is poor This is disconcerting when one

considers the importance of water as the universal solvent However, when the technical difficulties are evaluated, it becomes evident that these have been the deciding factor against an unequivocal answer A decisive answer awaits the reinvestigation of the problem by means of isotopic tracer technique

On morphological grounds, it has been assumed that the skin is impermeable to water (49) at least on short contact (7) because of the water shedding action of the surface layer of fat and lipids extending inward through the layers of cornified epithelia These lipids (cholesterol etc) are not hydrophobic, but hydrophilic, and upon prolonged contact enable the layers to take up water to a considerable degree, a phenomenon frequently observed as whitening of the palms and soles of the feet According to Rothman (6) this water imbibition of the keratin is enhanced by its acid nature (pH 5 5) This property of taking up water is lost in the layers of epidermis below the cornified (the "change over layers"—spinosum and granulosum) where Schmidtman (12) has shown the pH may be as high as 7 4. Here the protein being nearer to its isoelectric point should lose its tendency to take up water Rothman (6) advances the theory, therefore that the spinosum and granulosum layers may be considered the skin barriers to water While no experimental proof for this line of reasoning is forthcoming, it may be significant that the skin of the frog, which lacks these layers, is freely permeable to water

*Electrolytes* Although there is still difference of opinion, as in the case for water, the weight of evidence seems to favor the viewpoint that the skin of man and the higher animals is relatively impermeable to electrolytes

Attempts to determine whether the common electrolytes such as KCl, NaCl, $CaCl_2$ penetrate the skin have been faced with technical difficulties, chiefly due to the fact that it is necessary to demonstrate significant variation in the levels of substances already present in considerable quantities in the body tissues and fluids Crippa (105) claims to have found in one case following an hour's immersion of the body in a saline bath containing 11 85 grams Cl, 0 1081 gram Br and 0 04117 gram I per liter, a rise in the urinary excretion of chlorides of from 6 to 18 6 grams! Measurable increases in I and Br excretions also occurred This finding has never been confirmed with respect to the chloride, and can perhaps be explained by the entrance of chlorides into the body through other avenues than the skin Using Hediger's (44) method, Lehman (106) was unable to find measurable penetration of NaCl or $CaCl_2$ Burgi (45) was able to determine absorption of traces of chlorides and iodides, but only from highly concentrated solutions (10 to 27 per cent) This method is probably more suitable for analyses of the common electrolytes, but may not be sufficiently sensitive to record very small changes Using a somewhat similar "remainder analyses", Antinobin (107) showed uptake of chloride and iodide by the hands immersed in a normal solution of the salts The amounts were small but measurable. Using a new method, which depends on a change in the D C resistance of the skin as a measure of the amount of conducting electrolytes present in (and presumably passing through ?) the skin, Pomanskaya (108, 109) finds that the permeability decreases in the following order for the cations $K > Na > Li > Ca > Mg >$

Al, and for the anions I > Cl > $NO_3$ > $SO_4$ > citrate > salicylate Unfortunately, it is not indicated what amounts of the substances penetrated Stejskal (34) states that the skin is freely permeable to KI and that increased penetration occurs when the electrolyte is applied in the form of an ointment Gunther (110) reports penetration of KI, $NH_4I$, NaI, on the basis of a rise in the iodides of the urine Zwick (111), however, in some model experiments using skin freshly removed from the body was able to show that with LiI, and presumably also with iodides of other alkali metals, a splitting of the compound occurred, i e , the I penetrated and the Li remained behind Thus it would not be correct to assume penetration of the cations on the basis of finding iodides in the urine In other experiments where solutions of Li, Rb, Sr and Ba salts were applied to the living skin, Zwick (111) was unable to detect any traces of these metals in the urine by spectroscopic means This was confirmed by Kahlenberg (52) who immersed the feet of his subjects in hot foot baths containing the chlorides of Rb, Sr, Ba, Cs and Li No traces of these metals could be detected spectroscopically in the urine Whitehouse and Ramage (112) tested the permeability of the skin to LiCl The arm was immersed for an hour in warm solutions of LiCl ranging from 0 5 to 14 per cent Although they were able to detect 1 mgm of orally ingested Li in blood and urine by their spectroscopic method, not a trace could be detected after dermal exposure Because of the great sensitivity of spectroscopic analyses, this must be regarded for the present as the best evidence that the skin is for all practical purposes impermeable to electrolytes As to why the skin should be impermeable to electrolytes, the electrophysiological studies of Rein (80) have done much to advance a very plausible answer According to his studies, the skin behaves like a charged membrane with a negative charge on the outside Theoretically this membrane should be cation permeable and anion impermeable, and, therefore, in general, impermeable to electrolytes

*Gases* The literature is in general agreement that all true gases, and many vaporized and volatile substances, pass through the skin in an inward direction, CO appears to be an important exception It is doubtful that the avenues of entrance can be restricted to any one pattern such as the appendages (113) It appears that the process is one of simple diffusion across the entire skin which behaves like an inert membrane This diffusion is influenced by a number of factors (a) The tension or concentration difference of the gas on the two sides of the membrane (44, 114), (b) temperature (115), (c) solubility The last involves both water solubility (115, 116) and lipoid solubility Schwenkenbecher (117), in discussing the condition favoring passage of gas inward through the skin, regards lipoid solubility of great importance, citing the old model experiments of Filehne (100) According to this investigator the order of increasing lipoid solubility is H < CO < $O_2$ < N < $CO_2$ < $H_2S$ In the case of oxygen there is no doubt that penetration although small occurs Krogh (118, 119) found a range of 0 5 to 1 6 cc per square meter skin surface per hour for the pigeon and the eel whose systemic oxygen requirements are respectively 1600 and 30 cc per hour This lack of correlation between skin penetration and systemic requirement

prompted Krogh to consider the cutaneous uptake of $O_2$ as one of pure physical diffusion For man Zuelzer (120) has determined a maximum value of 0 7 cc $O_2/(m)^2$/hour which is only $\frac{1}{100}$ that of the respiration through the lungs It is very doubtful whether this small amount is of any physiologic significance. Goldschmidt et al (121), however, found that in unusual situations such as occlusion of the circulation of the arm, oxygen may diffuse into the skin from an oxygen filled plethysmograph and oxygenate the blood in the cutaneous vessels. Shaw, Messer and Weiss (122) have noted that oxygen is taken up by human skin from an atmosphere which contains less than 0 5 per cent and, furthermore, that there is never a passage of $O_2$ outward Elevated temperature favors this diffusion These observations indicate something more than simple diffusion for $O_2$, for the present the authors chose to regard this phenomenon as a reflection of local tissue oxidation, and of no general systemic significance

With adequate techniques (44, 45, 122), consisting of determining the disappearance of $CO_2$ from sealed containers in contact with skin, the passage of $CO_2$ inward through the skin has been well established In early work (123) proof of the penetration of $CO_2$ through the skin was attempted by measuring changes in alveolar $CO_2$, a technique fraught with serious error, as demonstrated by Liljestrand and Magnus (124) Hediger (44) showed that when a small glass bell cemented to the skin was filled with a $CO_2$-charged solution, $CO_2$ passed inward through the skin so long as the tension was higher than alveolar (40 mm ) and outward when the tension in the solution fell below this. He calculated that from hot natural waters charged with 700 to 800 cc $CO_2$ per liter, the whole body could take up as high as 200 cc $CO_2$/min ! This would constitute a large proportion of the turnover of $CO_2$ by the lungs, and is obviously fantastic, since it would lead to marked respiratory stimulation, which never occurs Kramer and Sarre (94) have measured the $CO_2$ diffusion inward through the skin at room temperature using Hediger's method The $CO_2$-charged spring water contained 88 volumes per cent $CO_2$ at a tension of 650 mm Hg Assuming a body surface of 2 $(m)^2$, these investigators found that penetration of $CO_2$ through the skin occurred at a rate which varied from 15 to 42 cc per minute with different individuals While these values are of a different order of magnitude from those of Hediger (44), Kramer and Sarre, nevertheless, confirm that with rise in temperature and $CO_2$ tension of the solution, very large increases in $CO_2$ diffusion do occur Shaw and Messer (115), in very careful work with a plethysmograph covering the entire arm, have determined that at above 8 5 per cent $CO_2$ the gas moves inward, below 8 5 per cent $CO_2$ the gas moves outward through the skin This equilibrium point is somewhat higher than Hediger's (44) $CO_2$ penetration is enhanced by both moisture (115) and elevated temperature (116) Lehman (106) has reported that the presence of salts seems to reduce the penetration of $CO_2$ from solution when applied to the skin after the method of Hediger There is an extensive literature on the penetration of $CO_2$ through the skin, it is mostly concerned with improvements of technique but does not materially change the concept of $CO_2$ penetration of the skin It seems, therefore, unprofitable to pursue the subject further

$H_2S$ and HCN have been extensively investigated because of the possibility of industrial hazard (44, 125, 126, 127, 128, 129) There is no divergence of results on HCN According to Walton and Witherspoon (130) it penetrates the intact skin and causes death to experimental animals exposed to from 5 5 mgm to 15 mgm per liter of air Guinea pigs were killed in 8 minutes following exposure of an area of belly skin only 1 inch in diameter, dogs after 47 minutes In some cases the hair was not even removed from exposure areas Schutze (131) confirmed the results on cats, and determined the concentration of gas below which no symptoms occurred to be about 0 4 per cent In one experiment on himself, systemic effects such as weakness occurred after 27-minute exposure of arm skin to 2 2 volumes per cent

For $H_2S$ the results have not been unequivocal Schutze (131) could demonstrate no systemic effect in cats and guinea pigs when one-fourth of the body surface, with hair removed, was exposed to 100 per cent atmospheres of $H_2S$ for 60 minutes Although he experienced severe burning and irritation followed next day by erythema and discoloration when his own arm was exposed to a 25 per cent atmosphere of $H_2S$, no systemic effects were noted Yant (132) reported that the whole body of man may be exposed to 2 per cent $H_2S$ for 30 minutes without skin discoloration, discomfort or systemic reaction Walton and Witherspoon (130) were able to poison guinea pigs in 38 to 45 minutes when about one-half of the body surface was exposed to 100 per cent $H_2S$, but in one dog, under similar conditions, no symptoms occurred Laug and Draize (17) were able to poison rabbits by exposing 9 3 per cent of their body area to pure $H_2S$ and the gas was detected in the expired air within 7 minutes but systemic toxicity was sometimes delayed A number of other investigators have reported positive findings with regard to $H_2S$ (113, 126, 128) It is necessary to reject the opinion of Rothman (6) that the toxic symptoms following skin exposure are caused by technical difficulties, such as leakage, leading to inhalation of $H_2S$ rather than by the gas penetrating the skin Much more pertinent, in interpreting positive results after long exposure to $H_2S$, are the considerations of possible injury to the skin There can be no doubt that under certain conditions $H_2S$ may act as a severe skin irritant The question then arises, how normal is this skin which is being permeated?

Behnke and Willmon (134) have determined the diffusion rates of N and He inward through the skin The technique involved placing the individual naked in a rubber sack with air tight neck piece, and measuring the respiratory output of the gas in question When the bag was filled with He at 700 mm pressure, from 40 to 60 cc of this gas diffused through the skin (1 7 $(m)^2$ surface) per hour at room temperature 27°C When the temperature was elevated to 35°C the diffusion rate increased to 170 cc per hour, and could be correlated with a rise in peripheral blood flow When the bag was filled with nitrogen the rate of diffusion of N was (after having first washed out body nitrogen), under the same conditions, only about half that for He

It is indicated that the human skin is permeable to radon in small amounts (135, 136) By testing the radioactivity of the expired air, it was determined

that more passed through the skin from warm water than from an air bath In all cases the permeability was of the order of 0 01 per cent, but was markedly increased by overlayering the skin surface with grease

Burgi (45) has reported that the skin is permeable to ammonia, but gives no details to show to what extent the skin may be injured (17) by this highly irritating gas

*Fats.* On theoretical grounds it may be assumed that the true fats and the non-saponifiable substances such as vaseline and mineral oil cannot pass through the skin. This is because the fats are fat soluble and water insoluble, a condition incompatible with the Overton theory which requires both lipoid and water solubility in order for a substance to move within and between the cells (Süssmann) (50) The explanation advanced by Rothman (6) that fats cannot pass through the skin because they exist in a colloidal disperse phase, and hence are unable to penetrate the cell walls, is probably not relevant Theoretically the lipoid substances such as cholesterol, lecithin and their derivatives, as well as the fatty acids, should be able to penetrate the skin because they are miscible in both fats and water There exists also the possibility that a true fat, in contact with the skin, may be subject to the action of skin lipases (137) and in this way attain hydrophilic properties

In general, it can be said that the literature is far from any consonance with the above theoretical considerations This is typified in the antipodal view points held by two schools of thought in Germany Latzel and Stejskal (138) claim that 150 to 200 grams olive oil can be inunctied daily and cite this as a means for cutaneous feeding of patients Wintornitz and Nauman (139) reject this entirely and find on the basis of iodized oil inunctions that not more than $\frac{1}{1000}$ of this amount can possibly penetrate The issue has been further confused by the use of inadequate methods Inunction, for example, of an oily or fatty substance into the skin until the surface seems to have lost all trace of it has been one of the commonest reasons for believing that penetration has occurred Unless it can be shown that the inunctied material or some derivative has actually entered the blood stream or subcutaneous tissues, it must be assumed that although within the appendages the material is still for all practical purposes "outside" the body Wild and Roberts (47) have applied the "difference" method to a study of vehicles for the inunction of mercury Weighed amounts of hydrous lanolin, lard and petrolatum were rubbed into the skin of the forearm and the excess carefully scraped off and again weighed It was thus found that 20 per cent of the lanolin, 15 per cent of the lard and only 10 per cent of the petrolatum was "absorbed", the order at least confirming less quantitative observations on the relatively poor absorption of the mineral base The difference between active inunction of a skin area and the passive application of a quantity of fat beneath a watch glass secured to the skin is demonstrated in the results reported by Bernhardt and Strauch (140) These investigators found no measurable loss of fat through contact with the skin, but their results may be in error because no account was taken of a possible compensatory weight gain of this fat depot by water uptake from the skin

Determination of fat or derivatives in the body tissues or excreta by chemical methods following skin exposure would seem to be the only means for obtaining an unequivocal answer to the problem The difficulties of this approach are twofold (a) The presence of an already large and variable lipid "mirror" in the blood, which would make small changes referable to a "fat stream" from the skin difficult to interpret (b) The extreme slowness with which fat depots subcutaneously introduced are absorbed (139, 140) Moser and Wernli (141) have reported an increase in the fat excretion in the urine following inunction of 20 to 30 grams' lard to the arms and chest In one case there was a 100 per cent increase in the absolute amount, in the other case no absolute increase, but a rise in concentration in the urine A nephelometric method was used, but insufficient data are given to establish the range of normal variation Both the positive results of Stejskal (142) and the negative results of Bernhardt and Strauch (140) and Winternitz and Naumann (139) depend on whether or not iodine is found in the urine following cutaneous application of iodopin, an iodized oil Regardless of the interpretation of the findings, it would appear that the results prove nothing because no allowance is made for possible fixation of small amounts of iodine that may have penetrated, and because the unwarranted assumption is made that the iodopin compound penetrates undecomposed through the skin In the older literature Lassar's (143) finding of fat droplets in the internal organs and blood after treating the skin of rabbits with turnip oil, olive oil, and cod liver oil, could not be confirmed by Winternitz (73) Sobieranski (144), without even recourse to control studies, has claimed that vaseline penetrates the skin on the basis of the finding of small amounts of unsaponifiable materials in the tissues!

Attempts have been made to study the permeability of the skin to fats by the use of histological and histochemical means While these methods have shown the probable avenues of entrance, no completely satisfactory evidence of absorbed fat below the rete malpighii and in the cutis has yet been presented Sutton (145, 146), one of the first to apply histological techniques, added the dyes fuchsin and scarlet (scharlach) to a number of fats and oils in order to trace their path into the skin after inunction The assumption was necessarily made that the penetration of the dye would go pari passu with its vehicle In no instance was dyed fat ever observed to pass directly into or through the skin and Sutton interpreted his experiments to mean that the skin was impermeable to fat Penetration, however, occurred into the appendages Under the most favorable conditions the entire lumina of the sebaceous glands were colored but never the base of the hair shaft On the basis of his observations, Sutton made a rough evaluation of the "penetration" abilities of a number of common fats and oils (a) Mineral oils and petrolatum—poor, slight coloring of skin surface (b) Vegetable oils—fair to good, coloring partly within the mouth of the shaft and sebaceous duct (c) Animal fats such as goose grease—excellent, coloring of entire lumen of sebaceous gland and upper half of hair shaft Unna and Frey (147) used India ink as tracer for white petrolatum and a water-in-oil emulsion (Nivea Creme) consisting of petrolatum or lanolin emulsified with a higher alcohol of the meta cholesterol series These investigators also could

find no evidence of the tracer within the cutis, but on the basis of penetration into the appendages confirmed the poor penetration of petrolatum but found excellent penetration of the "Nivea Creme" which they assumed was due to the water and emulsifying properties of the cholesterol The marker for these experiments was an unfortunate choice, it was not soluble, but merely suspended Thus the possibilities of separation from the vehicle can certainly be assumed to be greater With some variations, the histological technique has more recently engaged the attention of a number of investigators (38, 39, 65, 148) Of these, Strakosch (65) and Harry (148) confirm that the only visible penetration of fats is via the appendages. With perhaps better technique the loose upper layer of stratum corneum has frequently been seen to be colored, especially after lard, but never any layers below this It is interesting to note that Harry found that oleic acid was absorbed into the appendages better than the fats, perhaps this can be attributed to the hydrophilic group at one end of the molecule

Eller and Wolff (39) applied olive oil, cocoa butter, cod liver oil, hydrous lanolin, liquid petrolatum and petrolatum to the skin of rabbits In confirmation of the other investigators they found that the ascending order of penetration, as judged by the amounts found on the surface and within the sebaceous glands and hair shaft, was mineral, vegetable and animal fat The soft, oily material penetrated better than the solid Except in the case of the mineral bases, photomicrographs of the skin section showed a maximum amount of penetrating material in the shaft after four hours with a tendency for this to disappear after six hours The authors apparently did not wish to commit themselves concerning several sections which showed evidences of discrete fat masses within the cutis This would indicate complete penetration of the epidermis Unfortunately the authors did not use colored fats, but stained the skin sections to bring out the presence of the inunctod fats

Duemling (38) studied the effect of wetting agents on the penetration of fatty substances Unfortunately this investigator also stained the section rather than the fatty substance prior to application The criticism here is perhaps not as serious because relative rates of penetration were studied with and without the wetting agents The possibility, however, that the wetting agents might modify the distribution of the natural fats within the skin was not controlled but must be reckoned with According to Duemling there was little difference in the penetration of fats alone as measured in biopsy sections taken at 15 and 60 minute intervals No inunctod material was found very deep within the hair shaft In contrast, in the presence of wetting agents, fats were seen almost at the base of the hair shaft within 15 minutes After 30 minutes, fat had penetrated beyond the base of the hair shaft, and in fact to the base of the skin section (4 mm ) This is the only study which purports to show the penetration of fat actually into the cutis itself from the base of the hair follicles The importance of this observation, if substantiated, can hardly be over-emphasized Because of the fact that the normal skin does contain dispersed fat globules in those areas from which the sections were taken conservative judgment should await the preparation of sections made with colored fats.

*Carbohydrates* The information on this class of substances is meager In

evaluating such data as are available, it would appear at present that the weight of evidence for or against the passage of carbohydrates through the skin is equivocal As a class, the carbohydrates are water soluble or hydrophilic (starches) but lipoid insoluble (50)—properties incompatible with passage through an oil-water medium On the other hand, being non-electrolytes, one could at least expect that the skin would offer no electrical barrier to the carbohydrates as it does to electrolytes According to Chomkovic (149), passage of carbohydrates through frog skin occurs with the same facility as water, in mammals, however, Rothman (6) assumes that the same barrier to carbohydrates exists as it does to water The situation in the literature is about similar to that of the fats The same polemics have raged between the Stejskal (142) and Winternitz (150) schools, the former citing additional (151) evidence to prove that lactose excretion rises to 76 per cent of that injected, the latter (139) being unable to find sucrose in the urine after injection of large amounts, and citing the failure of Bauke (152) in obtaining any trace of mucic acid in the urine after 160 grams of lactose were injected in 4 consecutive daily injections

*Proteins and amino acids* There is evidence that minute quantities of protein may, under certain conditions, penetrate the skin and circulate in the blood To show this, Walzer et al (153, 154) have injected a cottonseed protein into the skin After a suitable interval, a second topical application is made at a place removed from the first Within a few minutes a reaction at the first injection site occurs This the authors consider proof that antigen must have circulated systemically Golowanoff (155) has reported that guinea pigs can be sensitized to horse serum by keeping a compress moistened with the protein in contact with the shaved skin After 14 days an injection of horse serum produces fatal anaphylactic shock Kimura (157) has reported that egg white in petrolatum, when injected into the skin of rabbits, can be detected by the production of antibodies in the blood within 7 to 10 days Owing to the delicacy of antigenic reactions, it is not surprising that the penetration of extremely minute quantities of protein through the skin can be detected By the same token, however, caution must be observed that no lesions in the skin occur It would appear likely, therefore, that the process of shaving or chemical depilation, as frequently practiced in animals, would not be suitable

While there is evidence that minute amounts of protein penetrate the skin, the claims of Stejskal (156) that gross amounts may be injected for nutritional purposes can hardly be taken seriously This investigator reported that protein in fairly large amounts could be injected into the skin by the use of a proprietary salve, "Dinutron", which contained 5 per cent egg white, 50 per cent fat and 37 per cent carbohydrate Repeated treatment of the skin made it more permeable to the protein, so that gram quantities (!) could be injected The criterion for penetration of protein was a rise in N excretion in a patient previously in N balance At best this analytical application is crude and no sensitization tests were made to check

With regard to the amino acids, which may be considered lipoid insoluble (50), there is an isolated report of the penetration of cystine (158) through the skin and its detection as a rise in sulfur in the urine

Because of the great interest in finding another route besides injection for supplying insulin to diabetics, numerous cutaneous studies have been made The evidence in general seems to indicate that insulin can pass through the skin and lower the blood sugar both in man and the rabbit The amounts required to produce effects comparable to subcutaneous injection are from 10 to 40 times as great (159, 160) In practically all cases it is necessary to pretreat the skin by a variety of means such as "stimulation" with heat, alcohol, ether or chloroform (161) or alkaline petroleum ether (162) Hermann and Kassowitz (101) in an extensive investigation concluded that cholesterol and fats on the skin surface were a chief barrier to the passage of insulin and established the rationale for the use of volatile solvents such as those mentioned for their solution and removal In addition they determined that alkalinizing the skin beforehand greatly facilitated insulin passage It is somewhat questionable whether the methods used for rendering the skin permeable to insulin have not in some cases rendered the skin abnormal It may be significant that Bruger and Flexner (163) in explaining their negative results conclude "that the absorption of insulin by the skin of rabbits is dependent upon the integrity of the integument the intact skin shows little or no absorption" This statement may also be applied to include chemical damage which is frequently not as evident Nevertheless, there is evidence that insulin also passes through the untreated skin from bland ointment bases such as "eucerin" (164) or lanolin (159) or even alone without any vehicle (161)

*Mercury and heavy metals* The greatest amount of attention has been focused on mercury because of its clinical application by cutaneous inunction in the treatment of venereal diseases The literature on the subject is enormous, and it would not be profitable to consider it in detail From the welter of earlier conflicting evidence, it can now be definitely stated that mercury passes through the skin in small quantities, and can be found in tissues, body fluids and excreta by routine chemical analysis (43, 50, 165, 166, 167) While the chemical methods have successfully shown that mercury penetrates the skin, they have not been suitable for showing how mercury enters The earlier histological work of Neuman (168) and Fuerbringer (169) indicated that the avenues for the entrance of mercury were exclusively through the appendages, chiefly the sebaceous glands and hair follicles Neisser and Siebert (170) confirmed this and were even able to show that metallic mercury seemed to penetrate much deeper into the hair follicle spaces than calomel, presumably because of its smaller state of subdivision Zwick (42), using metallic mercury ointment, showed that no particles of mercury could be discovered by microscopic examination direct, or by chemical reaction, either in the deeper layers of the stratum corneum or in the strata below The principal deposits of mercury were in the sebaceous glands and hair follicle shafts. On removing sections of inunctcd skin at intervals, he was also able to show that the bright, rounded globules seen at first tended to become angular and dull, as though chemical reaction had taken place Harry (148) inunctcd mercuric oxide in a lard vehicle and showed by sulfide treatment that mercury had lodged in the sebaceous glands Finally, Moncorps (43), while confirming all of the other work cited, reported that there was

evidence also of fine mercury particles in the interstitial lymph channels of the cutis If this latter observation could be confirmed, it would indicate that the penetration of mercury may not be limited exclusively to the pilosebaceous channels For the present, however, it would appear that the weight of evidence favors the latter mode of entrance almost exclusively

Early work of Brezina and Eugling (171) indicated that lead oleate, but not metallic lead, was able to permeate the skin from a lard-lanolin ointment base These investigators used the basophilic granule response as indicative that lead had entered The best evidence for the passage of lead through the skin of man, cat and guinea pig is based almost entirely on the painstaking chemical analyses of Süssmann (50) His frequently quoted figure for lead penetration of 0 1 to 0.2 mgm per square meter of skin per day, however, is undoubtedly too high because it is based on inadequate control analyses Compared to mercury, lead penetrates much less (178) and it may be assumed that this is due to the greater tendency of this metal to enter into insoluble combinations with the tissue proteins (50) Süssmann's histological investigations indicate that the probable avenue of entrance of lead is, as with mercury, through the appendages According to Tsunoda (41) the penetration of lead is directly into the cutis Since there is no theoretical reason for believing that lead would behave differently than mercury, this result must be questioned until confirmed by other workers

Recently, Schmid (172) has reinvestigated the permeability of human skin to diachylon (lead oleate) He finds, after the application of 10 grams to 1000 square centimeters of skin of the back, that the lead excretion averages 0.115 mgm in 24 hours and 0 270 mgm in 48 hours, indicating that on prolonged contact, there is progressive permeation of lead through the skin Penetration of lead is increased by salicylic acid, presumably through keratolytic action

According to Schmid and Winkler (173), copper penetrates the human skin from copper oleate applied in a lanolin-petrolatum base Following application of 10 grams to 1000 $(cm)^2$ of skin of the back, there is a progressive increase for several days in urinary copper excretion A marked increase results if the copper preparation is applied to damaged skin

Arsenic penetrates the skin Leva (174) has shown that after the exposure of the feet to a hot bath containing 19 mgm A's per liter, the element can be detected in the urine Macht (175) cites the case of a fatality resulting from the spilling of an arsenic-containing fluid over the legs and feet Fuchs (176) reports that the daily inunction of arsenic ointments for 14 to 37 days causes a urine excretion of As as high as 5 to 10 mgm per day Draize et al (179) have studied organic compounds of tri- and pentavalent arsenic incorporated in a variety of bases They found that the arsenic readily permeated the skin of rabbits, and could be detected in the urine within 24 hours

Finally, Komiyamas (177), on the basis of blackening of subcutaneous sulfur deposits beneath the site of inunction, considers this evidence for the penetration of bismuth from a salve

There is theoretical reason to expect that other heavy metals are capable of penetrating the skin

*Vitamins* There is evidence that vitamins A, B, C, D and K pass through the skin. In the case of $B_1$ (180) and C (181), the evidence coming from but one laboratory is as yet unconfirmed, indeed the complete lack of lipoid solubility of ascorbic acid makes this positive result rather surprising Vitamin A, being lipoid soluble, might be expected to pass through the skin, and the evidence for this although not extensive is uniformly positive Helmer and Jansen (182) were able to prevent severe vitamin A deficiency symptoms by skin application in rats, and this was confirmed by Eddy and Howell (183), who observed in addition that carotene was absorbed better than vitamin A Recently Mandel baum and Schlesinger (184), using the dark adaptation test, concluded that vitamin A, when applied as an ointment, can be absorbed through human skin in physiologically significant quantities Since the experiments of Hume, Lucas and Smith in 1927 (185) a considerable number of investigators have confirmed their findings that lipoid soluble vitamin D passes through the skin in sufficient quantities to exert curative effects (186, 187, 188, 189, 55) In fact there have been two reports where even symptoms of vitamin D poisoning have followed extensive application of a D-containing ointment to the skin of normal rabbits (190, 191) Recently (192) successful percutaneous therapy with vitamin K was reported in infants suffering from prothrombinopenia For additional review of data on vitamin passage through the skin, reference should be made to Eller and Wolff (193) Generally speaking, the problem of vitamin passage is an academic one, for in practice the amounts necessary for percutaneous therapy are considerably larger than for other routes of administration

*Sex hormones* Since the discovery of Zondek (194) that folliculin could pass through the skin of the mouse in sufficient quantity to exert physiological effects a number of investigators (195, 196, 197, 198) have confirmed and extended these observations Even though the sex hormones are lipoid soluble, incorporation in oily and fatty vehicles seems to slow up their absorption With estrogen, Zondek (199) found that it required 7 times as much to produce an effect quantitatively the same as when the vehicle was alcohol, ether or benzene, indeed with the latter vehicles there seemed to be little difference between cutaneous and subcutaneous administration This was confirmed by Emmens (200) It is possible that the volatile solvent vehicles render the skin more permeable by removal or modification of the natural lipoid matrix within the skin, and in this sense, the skin may be abnormal According to Zondek (199) progesterone in contrast to estrone is less easily absorbed through the skin Moore, Lamar and Beck (201), who investigated testosterone, found that this substance was readily absorbed by the skin from an ointment in sufficient amounts to maintain the accessory reproductive organs of castrate males in a normal reproductive state For more detailed review of the literature, reference should be made to Eller and Wolff (202) In general, it can be stated that, provided a volatile solvent vehicle is used, the percutaneous and subcutaneous administration of the sex hormones produce effects which are quantitatively of the same order

*Sulfonamides* penetrate the intact skin either from ointments (203 204, 205) or by iontophoresis of the sodium salts (206) According to Strakosch (203) there is no correlation between the penetration of sulfanilamide into the skin

of the guinea pig and the type of ointment base, whether water-in-oil or oil in water emulsion Woodard et al (205), on the other hand, found that the base was very important for absorption of sulfathiazole from the skin of rabbits, the order being as follows Fat in oil < water-in-oil < oil-in-water < propylene glycol Recently, MacKee et al (207) developed a histochemical method for demonstrating the presence of sulfonamides in the skin and other tissues With lanolin-water vehicle, only the surface and upper parts of stratum corneum showed sulfonamides, but with penetrasol (208) vehicle, sulfonamides were visualized in the hair shafts and even in the cutis

*Boric acid* This is an example of a lipoid-soluble, and feebly ionizing substance that is supposed to pass through the skin with great speed according to the frequently cited experiment of Kahlenberg (52, 209) When the feet were immersed in a warm bath containing 4 per cent boric acid in 30 per cent alcohol, boric acid could be detected in the urine within 50 seconds When $Na_2B_4O_7$ was substituted, no B could be found in the urine, the explanation for this being that the sodium salt ionized freely and was therefore lipoid insoluble It is unfortunate that such relatively high alcohol concentrations were used in the bath water, and it is therefore uncertain to what extent the permeability of the skin may have been abnormally modified Recent experiments of Pfeiffer (210) have failed to confirm the penetration of $H_3BO_3$ through the skin, it may also be significant that these investigators used no alcohol

*Iodine* An extensive literature has grown up on this controversial subject Nyiri and Jannitti (211) have reviewed in chronological sequence many of the references The reasons for the divergencies of opinion as to whether the skin is permeable to iodine or not, may be summed up as follows (1) inadequate chemical methods, (2) volatility of iodine, (3) injury to the skin by iodine or alcohol solvent, (4) fixation of iodine by tissues, (5) too short a period for urine collection, (6) failure to appreciate differences between the penetration of iodine and its salts Theoretically, since iodine is lipoid soluble, it should be able to penetrate the skin but, as in the case of some of the heavy metals, it may be retarded by its interaction with the tissue proteins (53) On the other hand, potassium iodide is lipoid insoluble and ionizes freely in solution. But Zwick (111), as mentioned above, has shown that salts of iodine may split up in the skin, the iodine finding its way into the body and leaving behind the cation The possibility of oxidation of iodides to iodine in the skin and the absorption of the elementary form (72, 36, 49) was recognized quite early, and Lehman (71) in reviewing some of the modern literature on iodine passage through the skin mentions the possibility that $H_2O_2$ resulting from the interaction between water and fat might form free iodine This would account in part for the enhanced penetration of iodine from potassium iodide ointments as compared with aqueous solutions It should be borne in mind that reaction of potassium iodide may also occur with the natural fats of the skin and this would serve to explain the uptake of iodine from potassium iodide baths (110, 105)

An extensive piece of work on the penetration of elementary iodine was

reported by Nyiri and Jannitti (211) Unfortunately these authors included no tabular material and a critical appraisal of their results is therefore not possible According to this investigation elementary iodine will penetrate the skin of dogs and rabbits when applied either in 5 per cent colloidal aqueous suspension or 3.5 per cent alcoholic tincture With precautions to prevent inhalation, iodides (never free iodine) were found in the urine within ½ hour whenever at least 160 mgm were painted on a sufficiently large surface 8–48 (inches)² The excretion of iodides was slow, 1 to 3 per cent of the amount applied to the skin of a dog was found in the urine within 3 days In pouch experiments in which a flap of live rabbit skin with partially intact blood circulation was used, penetration of iodides, and colloidal and alcoholic solutions of elementary iodine through the entire thickness of the skin was observed No matter in what form applied, only iodides were found in the Ringer solution into which the skin pouch was dipped The authors stress the striking fact that iodine also passed through the dead rabbit and dog skin pouches with the same facility " iodine finds its passage through the skin in the same amount irrespective of the condition of the tissue, whether the cells have a high or low vitality, or even are dead, and irrespective of the direction of penetration This seems to point to the fact that the activity of the cells is not indispensable as far as the passage of iodine through the skin is concerned In accordance with these results, we prefer to speak of the penetration of iodine through the surface of the skin and would reserve the absorption for the intradermal and hypodermal activity of the cells " It is unnecessary to emphasize the obvious importance of this observation and its acceptance must await confirmation by others With regard to the avenues of iodine entrance through the skin, there appears to be extant only the old histological work of Traube Mengarini (212) According to this, iodine penetrates through the appendages including the sweat glands, but not through the epidermis directly

*Salicylic acid* The literature on this subject is very large and it would not be profitable to review it in detail Reference should be made to Strakosch (213) who has considered some of the earlier investigations on salicylic acid, and to Hanzlik (214) who has reviewed the literature on the esters and compounds of salicylic acid The modern literature (215, 213, 57 216, 91) is in accord that salicylic acid and its esters pass through the skin with facility It is not entirely clear which of the two properties of this acid, its lipoid solubility or its keratolytic action, is the determining factor in permeation There appears to be fair evidence that the latter does increase its transfer through the skin (213, 57, 91), but this is denied by Rothman (6) and has been discussed under the section on skin function Presumably both factors are important, but, in the case of the esters, lipoid solubility is the more important The alkali salts of salicylic acid are unable to pass through the skin (6) They are strongly ionized and lipoid insoluble It has been assumed in the older literature (36) that when there was salicylate passage through the skin from a salt this was due to the liberation of free acid on the skin by the action of $CO_2$

## REFERENCES

### METABOLISM


(1) PILLSBURY, D M Ann Rev Physiol 2 151, 1940
(2) ROTHMAN, S AND P FLESCH Ann Rev Physiol 6 195, 1944
(3) WILKERSON, V A J Biol Chem 107 377, 1934
(4) *WILSON, R H AND H B LEWIS J Biol Chem 73 593, 1927*
(5) ASTBURY, W T J Chem Soc p 377, 1942
(6) HUGGINS, M L Chem Rev 32 195, 1943
(7) ASTBURY, W T Compt rend trav Lab Carlsberg 22 45, 1938
(8) ASTBURY, W T AND F O BELL Cold Spring Harbor Symposia on Quant Biol 6 109, 1938
(9) ASTBURY, W T Ann Rev Biochem 8 113, 1939
(10) STOVES, J L Trans Faraday Soc 38 254, 261, 501, 1942
(11) ROTHMAN, S J Investigative Dermatol 5 61, 1942
(12) PECK, S M. AND H SOBOTKA J Investigative Dermatol 4 325, 1941
(13) BAKER, A K , B E KLINE AND H P RUSCH Proc Soc Exper Biol Med 50 361, 1942
(14) MARTIN, G J , W A WISANSKY AND S ANSBACHER Proc Soc Exper Biol Med 47 26, 1941
(15) ROTHMAN, S J Investigative Dermatol 5 67, 1942
(16) FIGGE, F H J AND E ALLEN Endocrinology 29: 262, 1941
(17) *ROTHMAN, S Proc Soc Exper Biol Med 44 485, 1940*
(18) ROTHMAN, S AND P FLESCH Proc Soc Exper Biol Med 53 134, 1943
(19) FORBES, T E Endocrinology 30 456, 1942
(20) DAVIS, M E , M W BOYNTON, S FERGUSON AND S ROTHMAN J Clin Endocrinol 5 138, 1945
(21) RALLI, E P AND I GRAEF Endocrinology 32. 1, 1943
(22) HENDERSON, L M , J M MCINTIRE, H A WAISMAN AND C A ELVEHJEM J Nutrition 23 47, 1942
(23) DANN, F P , R C MOORE AND D V FROST Fed Proc 1. 107, 1942
(24) SCHAEFER, A E , J M MCKIBBIN AND C A ELVEHJEM J Biol Chem 143 321, 1942
(25) GROODY, T AND M E GROODY Science 95 655, 1942
(26) WRIGHT, L D AND A D WELCH Science 97 426, 1943
(27) FESSLER, A Brit J Dermatol and Syphilol 53. 201, 1941
(28) LEWIN, M L AND S M PECK J Investigative Dermatol 4 483, 1941
(29) MEIROWSKY, E Brit J Dermatol and Syphilol 52 205, 1940
(30) BAUMBERGER, J P , V DUNTZEFF AND E V COWDRY J Nat Cancer Inst 2 413, 1942
(31) MEDAWAR, P B Nature 148 783, 1941
(32) CORNBLEET, T Arch Dermatol and Syphilol 41. 193, 1940
(33) FOLIN, O , H C TRIMBLE AND L H NEWMAN J Biol Chem 75 263, 1927
(34) URBACH, E AND G SICHER Arch f Dermat u Syphilis 157 160, 1929
(35) TRIMBLE, H AND E W CAREY J Biol Chem 90 655, 1931
(36) URBACH, E AND K. RETITÖ Arch f Dermat u Syphilis 166 478, 1932
(37) MATSUMOTO, Y Mitt a d med Akad zu Kioto 7. 1307, 1933
(38) MATSUMOTO, Y Mitt a d med Akad zu Kioto 8 481, 1933
(39) NAHARA, K Jap J Dermatol Urol 35 31, 1934
(40) PILLSBURY, D M AND G V KULCHAR J Biol Chem 106 351, 1934
(41) MONCORPS, C , R M BOHNSTEDT AND R SCHMID Arch f Dermat u Syphilis 169 67, 1934
(42) URBACH, E , F DEPISCH AND G SICHER Klin Wchnschr 16 452, 1937

(43) Sellei, C and M Spiera Biochem Ztschr 296: 83, 1938.
(44) Schondorf as quoted by S Rothman Handb der Haut and Geschlechtskrankheiten I Part 2: 268 Berlin 1929
(45) Fahrig, C Ztschr f Krebsforsch 25: 146, 1927
(46) Gualdi, A. and N Baldino Riv de pat. sper 5: 318, 1930
(47) Pillsbury D M and T H. Sternberg Arch Dermatol and Syphilol 35 893, 1937
(48) Pillsbury, D M. J A M. A 96: 426 1931
(49) Nakamura, Y Biochem. Ztschr 173 258, 1926
(50) Eckstein, H C and V Wile J Biol Chem 69: 181 1926
(51) Unna, P G and L. Golodetz Biochem Ztschr 20: 469 1909
(52) Roffo, A H. Neoplasmes 7 344 1928
(53) Roffo, A H Bol Inst Med Exper Estud Cancer 8 32 1931
(54) Roffo A H J Physiol Path Gen 30 345 1932
(55) Engman M. F and D J Kooyman Arch Dermatol and Syphilol 29 12 1934
(56) Koffenhoeffer, R M J Biol Chem 116 321 1936
(57) Burtenshaw J M L J Hyg 42: 184 1942
(58) Wolbach, S B and O A Bessey Physiol Rev 22: 223 1942
(59) Vitamins in Dermatology Merck and Co , Inc , Rahway, N J
(60) Bessey O A. and S B Wolbach J A M A 110 2072 1939
(61) Elsom K. F Lewy and G W Haublin Am J Med Sci 200: 757, 1940
(62) Ball, E. G Cold Spring Harbor Symposia on Quant Biol 7: 100, 1939
(63) Denton, J Am J Trop Med 5: 173 1925
(64) Denton, J Am J Path 4 341 1928
(65) Eddy, W H. and G Dalldorf The avitaminoses. Williams & Wilkins Baltimore Md 1941
(66) Elvehjem C A Physiol Rev 20: 249, 1940
(67) Spies, T D Quoted in Cecil s Textbook of medicine 5th ed W B Saunders, Philadelphia, 1940
(68) Schaeffer, A. E J McKibben and C A Elvehjem J Biol Chem 144: 679, 1942
(69) Moore, R. A , T D Spies and Z h Cooper Arch Dermatol and Syphilol 46: 100 1942
(70) Spies, T D J A M A 115 839 1940
(71) Sullivan, M. and J Nichols J Investigative Dermatol 3 317 1940
(72) Atopol, W and P Unna Arch Path 33: 241 1942
(73) Howard, C P and E S Mills Oxford Medicine Univ Press 3: 161 1928
(74) Sullivan, M and J Nichols Arch Dermatol and Syphilol 45 295 1942
(75) Wooley D W J Nutrition 28: 304 1944
(76) Waddell, W W Jr and D M Guerry J Pediat 15 802 1939
(77) Burr G O and M M Burr J Biol Chem 82: 345, 1929
(78) Presnell, A K J Biol Chem 121 5 1937
(79) Way, S C and A Memmesheimer Arch Dermatol and Syphilol 41: 1086 1940
(80) McSwiney, B A Proc Roy Soc Med 27: 839, 1934
(81) Couraud J Compt rend soc biol 118: 155 1935
(82) Whitehouse, A G R Proc Roy Soc London B 117: 139 1935
(83) Tennent D M and R H Silber J Biol Chem 148: 339, 1943
(84) Sargent, F P Robinson and R. E Johnson J Biol Chem 153 285 1944
(85) Bernstein R. E Nature 140 684 1937
(86) Hardt, L. L and E U Still. Proc Soc Exper Biol Med 48: 704 1941

PERMEABILITY

(1) Pinous F Jadassohn s Handbuch der Haut und Geschlechtskrankheiten I Part 1: 105 Berlin 1927

(100) FILEHNE, W Arch Internat Pharmacodyn et de Therap 7 133, 1900
(101) HERMANN, S AND H KASSOWITZ Arch f exper Path u Pharmakol 179 524, 529, 1935
(102) MILBRADT, W Ztschr f d ges exper Med 87 745, 1933
(103) SPITTA, O Arch f Hyg 36 45, 1899
(104) WHITEHOUSE, A G R AND H RAMAGE Proc Roy Soc London B 111: 412, 1923
(105) CRIPPA, J F Wien klin Wchnschr 40 879, 1927
(106) LEHMAN, G Arch Internat de Pharmacodyn et de Therap 55 331, 1937
(107) ANTINOBIN, A Arch Internat de Pharmacodyn et de Therap 31 351, 1926
(108) POZNANSKAYA, N B J Physiol U S S R 28 323, 1940
(109) POZNANSKAYA, N B Bull biol med exper U S S R 4 477, 1937
(110) GUNTHER, G Prag Arch f Tiermed Series A, 55, 1926
(111) ZWICK, G K Korrespondenzbl Schweiz Arzte 47 1319, 1917
(112) WHITEHOUSE, A G R AND H RAMAGE Proc Roy Soc London B 113 42, 1933
(113) FLURY, F Festschrift-Zannger 2 836, 1935
(114) SHAW, L A AND A C MESSER Am J Physiol 98 93, 1931
(115) SHAW, L A AND A C MESSER Am J Physiol 95 13, 1930
(116) HEDIGER, S Münch med Wchnschr 77 1321, 1930
(117) SCHWENKENBECHER, A Münch med Wchnschr 80 1091, 1933
(118) KROGH, A Skand Arch Physiol 15 328, 1904
(119) KROGH, A Skand Arch Physiol 16 348, 1904
(120) ZUELZER, G Ztschr klin med 53 403, 1904
(121) GOLDSCHMIDT, S , B MCGLONE AND J S DONAL J Med Sci 187 586, 1934
(122) SHAW, L A , A C MESSER AND S WEISS Am J Physiol 90 107, 1929
(123) WINTERNITZ, H Deutsch Arch klin Med 72 258, 1901
(124) LILJESTRAND, G AND R MAGNUS Pflüger's Arch 193. 527, 1922
(125) HUNT, R Hefter's Handbuch der Exper Pharmakologie 1 723, Berlin 1923
(126) FLURY, F AND R WALTHER Arch Gewerbepath u Gewerbehyg 11 311, 1942
(127) MÜLLER-HESS, B Münch med Wchnschr 89 492, 1942
(128) HETZEL, K W Gasschutz u Luftschutz 2 236, 1932
(129) FAIRLY, A , E C LINTON AND F E WILD Hyg J 34 283, 1934
(130) WALTON, D C AND M G WITHERSPOON J Pharmacol and Exper Therap 26 315, 1925
(131) SCHUTZE, W Arch f Hyg 98 70, 1927
(132) YANT, W P Am J Public Health 20 598, 1930
(133) OIVIN, I A AND A I SUSLOVA Bull Biol exper U S S R 9 316, 1940
(134) BEHNKE, A R AND T L WILLMON Am J Physiol 131 627, 1941
(135) JANITZKY, A , W RASCHIG, O STEINKE AND W WICHMAN Klin Wchnschr 12 1692, 1933
(136) WAGNER, R Sitzber Akad Wiss Wien Math-naturw Klasse Abt 1 1a 143 521, 1934
(137) ROTHMAN, S AND F SCHAAF Jadassohn's Handbuch der Haut und Geschlechskrankheiten I Part 2 Berlin 1929
(138) LATZEL, R AND K STEJSKAL Wien klin Wchnschr 39 1219, 1926
(139) WINTERNITZ, H AND H NAUMAN Deutsch med Wchnschr 55 1828, 1929
(140) BERNHARDT, H AND C B STRAUCH Ztsch f klin Med 106 671, 1927
(141) MOSER, H AND A WERNLI Pharmaceut Zentralhalle Dtschland 69 401, 1928
(142) STEJSKAL, K Deutsch med Wchnschr 56 491, 1930
(143) LASSAR, O Virchow's Arch 77. 157, 1879
(144) SOBIERANSKI, W Arch f exper Path 31 329, 1893
(145) SUTTON, R L Monatsh f. prakt Dermatol 43 371, 1906
(146) SUTTON, R L Brit Med J 2 225, 1908
(147) UNNA, E AND W FREY Dermatol Wchnschr 88 327, 1929
(148) HARRY, R G Brit J Dermatol and Syphilol 53 65, 1941

(149) Chomkovic, G Pflüger's Arch 211: 668 1926
(150) Winternitz H Deutsch med Wchnschr 56: 492, 1930
(151) Strejskal K Wien. med Wchnschr 79 863, 1929
(152) Bauke, E E Deutsch med Wchnschr 56 1860, 1930
(153) Walker A Arch Dermatol and Syphilol 41: 692 1940
(154) Walker, A and S S Sack Arch Dermatol and Syphilol 49: 427, 1944
(155) Galowanoff, M Compt Rend Soc Biol 94 6, 1926
(156) Strejskal, K. Wien klin Wchnschr 77: 1358, 1927
(157) Kimura, G Oriental J Diseases Infants 28 15 1940
(158) Scoz G Boll Soc Ital Biol Sper 8 240, 1933
(159) Telfer, S V Brit. Med J 1: 715, 1923
(160) Wallgren, A Münch med Wchnschr 71: 10, 1924
(161) Wilkoewitz K and F Lenuweit Ztschr f klin Med 122: 621, 1932.
(162) Pribram H Klin Wchnschr 14: 1534 1935
(163) Bruger, M. and J Flexner Proc Soc Exper Biol and Med 35: 429, 1936
(164) Kingsepp G and L Talli Klin Wchnschr 18 1823, 1940
(165) Wile, U J and J A Elliott J A M A 68: 1024 1917
(166) Cole H N, J A Gammel J E Rauschkolb, N E Schreiber and T Sollmann Arch Dermatol and Syphilol 17 625 1928
(167) Cole H N H F DeWolf N E Schreiber, T Sollmann and J V Cleve Arch Dermatol and Syphilol 27: 1 1933
(168) Neuman I Wien med Wchnschr 21: 1209, 1233 1255 1871
(169) Fuerbringer, P Arch f patholog Anat u Physiol 82: 491, 1880
(170) Neisser A and C Siebert Med Klin 1: 9 1904
(171) Brezina, E and M Eugling Hyg Rundschau 23: 491, 1913
(172) Schmid R. Klin Wchnschr 21 158 1942
(173) Schmid R and J Winkler Klin Wchnschr 17 559, 1938
(174) Leva J München med Wchnschr 76 1368, 1929
(175) Macht D I Sammlung von Vergiftungs Fällen 1 103, 1930
(176) Fuchs L Deutsch Ztschr f d ges gericht Med 19 280, 1932
(177) Komiyamas, T Klin. Wchnschr 4 2012 1925
(178) Laug, E P and F Kunze Unpublished observations
(179) Draize J H S Berman and R B Lang Unpublished observations.
(180) Kasahara M, J Hayashi T Yokonowa and R. Furumi Klin. Wchnschr 17: 939 1938
(181) Kasahara M and K. Kawashima Klin Wchnschr 16 185, 1937
(182) Helmer, A C and C H Jansen Studies Inst. Divi Thomae 1: 1, 10 1937
(183) Eddy, W H and J L Howell N Y State J Med 39: 406, 1939
(184) Mandelbaum, J and L Schlessinger. Arch Dermatol and Syphilol 46: 431 1942
(185) Hume E M., N S Lucas and H H Smith Biochem J 21 362, 1927
(186) Von Mallinckrodt Haupt, A S Ztschr f Vitaminforsch 4 10 1935
(187) Fodor, M E Ztschr f Vitaminforsch 3 241 1934
(188) Astrowe P S and R A.Morgen Am J Diseases Children 49: 912 1935
(189) Helmer, A C and C H Jansen Studies Inst Divi Thomae 1: 83 1937
(190) Gordonoff, T and S Zurukzoglu Arch f Hyg 109 361, 1933
(191) Moncorps, C, H Droller and C E Carter. Münch med Wchnschr 80: 1239 1933
(192) Vollmer H C Abler and H S Altman Am J Diseases Children 64: 462 1942.
(193) Eller, J J and S Wolff J A. M A 114 2002, 1940
(194) Zondek, B Klin Wchnschr 8: 2229, 1929
(195) Loeser A A J Obstet Gynecol Brit Empire 44 710, 1937
(196) Sakanoue K. Japan J Obstet Gynecol 22 100, 1939
(197) Baer, H L J Investigative Dermatol 2: 15 1939

(198) SALMON, U J Proc Soc Exper Biol Med **38** 481, 1938
(199) ZONDEK, B Lancet **1**· 1107, 1938
(200) EMMENS, C W J Endocrinol **2** 368, 1941
(201) MOORE, C R, J K LAMAR AND N BECK J A M A **111** 11, 1938
(202) ELLER, J J AND S WOLFF J A M A **114** 1865, 1940
(203) STRAKOSCH, E A AND W G CLARK Am J Med Sci **205** 518, 1943
(204) STRAKOSCH, E A AND W G CLARK Am J Med Sci **206** 610, 1943
(205) WOODARD, G, C D WRIGHT, O L EVENSON, R R OFNER, D S KRAMER, P M JENNER AND V D JOHNSON Fed Proc **3** 87, 1944
(206) CLARK, W, E A STRAKOSCH AND C NORDLUM Proc Soc Exper Biol Med **50** 43, 1942
(207) MACKEE, G M., F HERRMANN, R L BAER AND M B SULZBERGER J Lab and Clin Med **28** 1642, 1943
(208) HERRMANN, F, M SULZBERGER AND R L BAER Science **96** 451, 1942
(209) KAHLENBERG, L AND N BARWASSER J Biol Chem **79** 405, 1928
(210) PFEIFFER, C C, L F HALLMAN AND I GERSH J A M A **128** 266, 1945
(211) NYIRI, W AND M JANNITTI J Pharmacol and Exper Therap **45** 85, 1932
(212) TRAUBE-MENGARINI, M Arch Anat u Physiol 1892, Suppl 1
(213) STRAKOSCH, E Arch Dermatol and Syphilol **47** 16, 1943
(214) HANZLIK, P Medicine **5** 197, 1926
(215) BROWN, E W AND W O SCOTT J Pharmacol and Exper Therap **50** 32, 373, 1934
(216) MONCORPS, C Arch f exper Path u Pharmakol **163** 26, 1931

# MECHANISM OF THE DEVELOPMENT OF OBESITY IN ANIMALS WITH HYPOTHALAMIC LESIONS[1]

JOHN R BROBECK

*Laboratory of Physiology Yale University School of Medicine New Haven, Connecticut*

The obesity which follows hypothalamic injury holds the interest of clinicians and of physiologists alike, because it is one of the few types of obesity of known etiology, and because it can be produced in laboratory animals for experimental study The injury responsible for the obesity may be the result of any one of several different processes In the monkey, dog, cat and rat, it has been produced either by surgical means, or electrolytically with the aid of the Horsley-Clarke sterotaxic instrument, while in man it is usually the result of some neoplastic or infectious disease involving either the diencephalon or its near neighbor, the hypophysis Whatever the immediate cause of the hypothalamic lesions, their consequences appear to be almost identical in every species studied, and it is therefore generally agreed that what has been learned upon the lower animals may be applied directly to the human syndrome

One of the most remarkable characteristics of this type of obesity is the extreme rapidity with which the individual gains weight In questioning the patient, the examiner often discovers that the patient had previously been of normal size and weight, that the obesity began rather abruptly, and that its onset may have coincided with the appearance of other signs and symptoms of a disease process near the base of the brain Each of these features was recognized by Mohr (1840) who first described this obesity, and consequently his report may be used to illustrate the usual clinical course of the disease. His patient was a 57 year old gardener's wife who had become obese within a space of one year For six years she had complained of vertigo and periodic headaches, and for three years she had suffered also from "mental deterioration" In her last year she became more and more incapacitated by specific neurologic deficits (both sensory and motor), her behavior became careless and childlike, and she became extraordinarily obese (her weight is not reported) She died rather unexpectedly, and the autopsy revealed a pituitary tumor, large enough to have eroded the sella turcica and to have distorted the base of the brain all the way from the anterior margin of the hemispheres to the pons varoli.

In laboratory animals with experimentally produced lesions of the hypothalamus a dramatic gain in weight has been observed within a few hours following the operation, an adult female rat, for example, may increase its body weight by as much as 15 per cent within the first 24 hours, and may then continue to gain for several weeks at a rate ten times the normal rate of gain. This rapid gain is not indefinitely maintained, and the animal eventually reaches a plateau

[1] The experiments described in this paper from the Department of Physiological Chemistry and the Laboratory of Physiology, Yale University, were aided by grants from the Markle Foundation and from the Fluid Research Fund and the George H Knight Fund of the School of Medicine Yale University

where its weight may remain relatively constant for many months (Keller and Noble, 1936, Hetherington and Ranson, 1940, Brobeck, Tepperman and Long, 1943a) Using the terminology of Rony (1940), Brooks (1945a,b) has designated the period of rapid gain as the dynamic phase and the plateau as the static phase of the obesity

From the anatomical or pathological point of view, the carcass of an obese animal is remarkable chiefly because of the great quantity of surplus fat contained in it Smith (1927) found this fat to amount to as much as 74 per cent of the total carcass weight (cf Hetherington and Weil, 1940) The depots of the subcutaneous tissue, the omentum and the mesenteries of the viscera are filled with fat, as are the perirenal tissues, the pericardium and, in fact, every location where adipose tissue normally exists The liver is yellow, may be double the normal size, and in the fed state may contain twice the normal percentage of ether-extractable lipid, which means that the total quantity of hepatic fat may be approximately four times the normal amount Plasma lipid concentrations are likewise elevated, especially in the fed state, and this elevation and the increased quantity of liver fat have been interpreted as evidence that the rate of turnover of lipids is abnormally high (Tepperman, Brobeck and Long, 1943) Though it should be possible to measure the rate of this turnover by the use of isotopic elements, such a measurement apparently has not been made, and the significance of the high lipid levels remains somewhat conjectural Likewise uncertain is the significance of the cirrhosis of the liver observed by Graef, Negrin and Page (1944) in certain obese dogs with combined hypophysial and hypothalamic injury If it is true that the rate of lipid turnover is elevated in this type of obesity, the animals might well use in this turnover large amounts of compounds such as choline which participate in lipid metabolism in the liver (McHenry and Patterson, 1944) The cirrhosis observed by Graef and his collaborators, then, might have been the result of a relative deficiency of lipotropic substances in the diet The problem appears to be worthy of further investigation for its possible contribution to the understanding of the pathways of lipid metabolism as well as for its application to the problem of obesity

Although complete histological descriptions of the organs and tissues of obese animals with lesions restricted to the hypothalamus have not been published, there is reason to believe that several different types of abnormality may be present The gastro-intestinal tract shows a generalized dilatation and hypertrophy which have been tentatively attributed to the unusual feeding habits of the rats in which the condition was noted (see below) The obese rat may also exhibit conspicuous abnormalities of kidney function and structure, characterized by the presence of albumin, erythrocytes and casts in the urine, and by hyalinization of the renal glomeruli, dilatation and necrosis of the tubules, and a generalized increase in the amount of connective tissue around the nephrons (Brobeck, Tepperman and Long, 1943a) Hypoplasia of the gonads and genitalia has been frequently observed in human patients as well as in experimental animals (Babinski, 1900, Fröhlich, 1901, Bailey and Bremer, 1921a, b, Hetherington and Ranson, 1942b, and others) This hypoplasia was at first assumed to be an

evidence of anterior lobe deficiency, more recently, however, similar abnormalities of the reproductive organs and of mating behavior have been observed in animals in which the anterior lobe was not directly injured, and there is a growing tendency to attribute regulation of reproductive functions to the hypothalamus (Dey, 1943, Bard, 1940) Just how the hypothalamus may control sexual functions remains to be discovered, and lacking further information, one cannot properly evaluate the significance of the genital hypoplasia present in the obese animal or patient. Brooks and his collaborators (1942) and Hetherington and Ranson (1942 b) have suggested that the two conditions, viz , obesity and genital hypoplasia, are not necessarily related, since certain lesions apparently produce one without the other But on the other hand, Bailey and Bremer (1921b) were of the opinion that the one condition may induce the other, i e , that obesity in some way alters the growth and function of sexual organs, and clinical literature already contains data to support their hypothesis, since obese women with irregular menstrual cycles or with amenorrhea have been found to recover normal cycles when they lose weight as the result of dietary restriction (Evans, 1940)

In order to complete this description of the obese animal, several other types of abnormality should be mentioned although their significance is not at all clear Changes in the skin and fur have been described, especially in the rat, where the fur apparently deteriorates in appearance because the animals are too obese to groom their coat as normal rats do (Hetherington, 1940, Graef, Negrin and Page 1944) Several investigators have also noticed that their obese animals readily succumb to vague, apparently nonspecific diseases in which the progress of the obesity is either temporarily or permanently interrupted (Hetherington and Ranson, 1940, Brooks, 1945a, Ingram, 1945) While present knowledge of the biology of these animals leaves much to be desired, the possibility that they may require relatively large amounts of the various accessory food factors appears to be worthy of consideration Their total energy exchange, which is the basis upon which vitamin requirements are usually estimated, is known to be much greater than normal (see below), and some of these disorders which appear to be nonspecific diseases may well be the result of certain types of dietary deficiency

In addition to the physical characters just described, abnormalities of behavior, especially hyperirritability and hyperexcitability have been reported Hetherington and Ranson (1942 c), referring to rats with lesions situated at the level of the pituitary stalk, wrote as follows "Often rats with large lesions in this region of the hypothalamus will run almost continuously in an automatic almost frenzied fashion for several hours after they awake from the anesthetic This period may be succeeded by another lasting several days when the animals will seem to be stuporous, but will respond with exaggerated violence to slight tactile stimuli. After the acute phases of the postoperative period are past the rats are rather lethargic, though this characteristic usually does not appear to partake of somnolence or a lack of alertness They are, in fact, generally somewhat irritable and *excitable for a number of weeks With handling the rats after a time often* lose this touchiness to a certain extent (It should be mentioned, in passing

that more recently operated rats with lesions in the caudal hypothalamus have displayed neither the acute hyperactivity nor the later hyperirritability of the rats just described)" The observations of Krieg (1938) and of Brooks (1945c, 1946) are in general agreement with Hetherington and Ranson's description Wheatley (1944), working in Ingram's laboratory, undertook a study of the affective behavior of cats with hypothalamic lesions, and obtained certain animals which, "in the presence of an observer, continually showed all the physical manifestations which have been observed in normal cats when provoked to rage, i e , mydriasis, horripilation, defecation, urination, vocalization of anger, spitting, lashing of the tail and a kyphotic attitude They would attack the observer in a vicious, aggressive manner, biting and clawing when attempts were made to pet them or when other innocuous stimuli were applied" Ingram (1945) studied additional animals of this type and observed, "Postoperatively (they) have a striking change in temperament ranging from unfriendly to outright savage." Several of the savage cats became obese, and there appears to have been some correlation between the change in temperament and the obesity Nevertheless, Hetherington and Ranson (1942c) observed no hyperexcitability in rats which became obese following the production of lesions of the posterior hypothalamus, and the obese rats prepared in the laboratory of Long (unpublished) were not difficult to handle Rather than irritability, the latter appeared to show apathy and indifference, as Bailey and Bremer (1921b) and as Smith (1927) had previously observed

*Irrevelevant nature of injury to the pituitary* The recent work of Hetherington clarified one of the most confusing and controversial issues ever to arise in experimental physiology, namely, the rôle of the anterior lobe in the development of obesity Hetherington (1943) has shown that hypothalamic lesions will produce obesity whether the anterior lobe is present or has been removed, and further, that no amount of pituitary damage will induce obesity when the hypothalamus is intact His experiments clearly indicate that the long-held opinion that the obesity of "Fröhlich's syndrome" is a sign of anterior lobe insufficiency is incorrect Because of the importance of Hetherington's conclusions, a brief review of the history of this problem appears to be in order here

Although Mohr published the first known description of a case of hypothalamic obesity in 1840, no one appears to have inquired into the cause of the obesity until early in the twentieth century, when the papers of Babinski (1900), Fröhlich (1901) and Erdheim (1904) appeared Then within a space of four years there arose what eventually became a classic controversy Fröhlich, recognizing that the obesity was usually associated with tumors of the hypophysis, considered the condition to be an endocrine disease, a sign of hypopituitarism But three years later Erdheim expressed a dissenting opinion, as follows "We agree with Fröhlich that pathological obesity is of frequent occurrence in (patients with) hypophysial tumors It seems to us, however, that he is unjustified in his interpretation that a change in function of the hypophysis as an endocrine gland is responsible for the adiposity We further believe that the reason for it must be sought in the tumor's influence—be it injury or stimulation

— on the region of the base of the brain" (p 689) Erdheim based his opinion upon the following facts 1 In certain cases of this obesity the tumor arises from structures other than the hypophysis and leaves the gland relatively undamaged 2 No particular type of tumor is associated with the development of obesity 3 Adiposity and acromegaly may occur together 4 Tumors which do produce adiposity always involve the base of the brain, although admittedly not all tumors which damage the brain produce obesity Erdheim concluded his paper, however, with a statement admitting that the exact region of the brain which must be affected to produce the disease was unknown to him

The appearance of conclusive experimental evidence in support of Erdheim's position was long delayed, since the rôle of the anterior lobe as a regulator of metabolic processes was first to be revealed, while the etiology of this experimental obesity remained a more or less subordinate and incidental problem When the obesity was produced in experimental animals, it was produced by operations upon the pituitary gland, and it was therefore considered to be a sign of hypophysial disturbance by those who believed that they had performed their operations without injuring the overlying hypothalamus (Crowe, Cushing and Homans, 1910, Bell, 1917, Dott, 1923) But the conclusions of this neurosurgical school were challenged by another group of investigators who found that operations upon the pituitary resulted in obesity only in those animals in which there was histological evidence of injury to the hypothalamus. Aschner (1912) was evidently the first to make this observation in laboratory animals, although general acceptance of his conclusions by other investigators appears to have been delayed by his attempt to distinguish between obesity as it develops in hypophysectomized puppies and as it occurs in adult dogs with hypothalamic lesions Nevertheless, later study generally confirmed his conclusions Camus and Roussy (1913, 1920, 1922) found that neither partial nor complete removal of the anterior lobe produced either obesity or diabetes insipidus in the dog unless the base of the brain was also injured, and their observations upon obesity were confirmed, in turn, by Smith (1927, 1930) upon rats in which an attempt had been made to compare the results of two different operations The former operation, hypophysectomy by a parapharyngeal approach produced cessation of growth, loss of weight, weakness, cachexia, and atrophy of the thyroid, suprarenal cortex and gonads, the latter operation, the injection of chromic acid into the region of the hypothalamus and pituitary gland using a subtemporal approach brought about obesity and genital dystrophy

In the meantime obesity had been produced in one or perhaps two dogs by hypothalamic injury alone The animals in question were described by Bailey and Bremer (1921a, b) among a series of dogs in which operations had been performed upon the base of the brain by way of the subtemporal approach perfected by Harvey Cushing The authors were primarily interested in the question of the origin of diabetes insipidus, but they obtained at least one unquestionably obese dog in which no direct encroachment upon the hypophysis could be detected by microscopic study of sections from that organ Until 1940 this remained the only case of true hypothalamic obesity known to have been reported in the

experimental literature It remained for Hetherington and Ranson (1939, 1940) to show that obesity regularly follows the production of lesions restricted to the hypothalamus in the rat, and to rule out the possibility that anterior lobe deficiency is in any way necessary for the development of this condition Where others failed or met with only limited success, Hetherington and Ranson succeeded because they used the Horsley-Clarke stereotaxic instrument (modified by Clark (1939) for use on the rat), with which discrete electrolytic lesions may be placed at predetermined locations within the brainstem Their results have been confirmed in other laboratories in the rat (Brobeck, Tepperman and Long, 1943a, Brooks, 1945a, b, 1946), the cat (Wheatley, 1944, Ingram, 1945) and the monkey (Brooks, et al , 1942, Ruch, Patton and Brobeck, 1942) Hetherington and Ranson (1942a) were able to show that the development of the obesity is independent of pituitary damage, since the condition can be produced not only in rats with uninjured hypophyses, but also in rats from which the hypophysis has been removed either 10–11 weeks before, or one week after the production of the hypothalamic lesions Their hypophysectomized rats with hypothalamic lesions exhibited characteristic dwarfism in addition to obesity The latter condition, therefore, is not directly related to the level of pituitary function, or, in the words of Hetherington (1943) "Since neither total nor partial hypophysectomy produces adiposity, or prevents its appearance after hypothalamic damage is done, it is not likely the hypophysis is involved in the production of obesity often associated with injury to structures in the pituitary region Hypothalamic disorder appears to be the sole responsible factor "

In view of the fact that the papers of Fröhlich (1901) and Erdheim (1904) began a controversy which endured for almost 40 years, a recent statement by Fröhlich (1940), made at the 1939 meeting of the Association for Research in Nervous and Mental Disease, is of considerable interest "The discussions that I have attended the past two days have established the fact that we were wrong in 1901, that it was not the pituitary body, but the hypothalamus, but then I must remind you that all we knew at that time was that the hypothalamus was an anatomical region lying beneath the thalamus Everything was in darkness like the gray substance, the tuber cinereum Hence, I attributed this syndrome to a tumor of the hypophysis without acromegaly Since the first description, many cases have been recognized" Fröhlich might have added that many cases have been not only recognized, but also described in medical literature, although with perhaps one or two exceptions[2] the more recent reports have added little to the picture presented forty years ago by Frohlich and Erdheim

*Localization of effective hypothalamic lesions* In only a very few of the published reports dealing with experimental obesity have the lesions been well enough described to permit identification of the cell groups concerned in the pathogenesis of this condition The obese dog (dog 10) observed by Bailey and Bremer (1921b) suffered two lesions of the posterior hypothalamus between the level of the stalk and the anterior border of the mammillary bodies, but from

[2] A notable exception is the critical survey by Bruch (1939), who has published an English version of Fröhlich's original paper

their description a more specific localization cannot be given The papers of Camus and Roussy (1913, 1920, 1922), Grafe and Grünthal (1929), Smith (1927 and 1930), Crooke and Gilmour (1938) and Biggart and Alexander (1939) contribute only limited information in this regard, either because exact descriptions of the lesions were not given or because the authors were unable to correlate the site of the injury with the appearance of obesity in the animals of their series The latter statement applies particularly to the papers by Crooke and Gilmour and by Biggart and Alexander, in both the lesions were said to involve the anterior hypothalamus, but other animals with what appeared to be similar lesions failed to gain extra weight. Heimbecker, White and Rolf (1944) have suggested that the paraventricular nucleus, especially its caudal portion, is concerned in some way with "fat metabolism " and that lesions which interrupt axons from cells of this nucleus produce obesity They report a 'good correlation" between injury to this nucleus and weight gain in their dogs Unfortunately, the free-hand lesions which they made damaged many other nuclei and interrupted many fibers in addition to those from the paraventricular nuclei, and hence their conclusions are not as convincing as they might be Hetherington (1941, 1944), on the other hand, placed discrete, well localized lesions in almost all parts of the hypothalamus of rats, and found that large lesions of the anterior or dorsal hypothalamic areas, or of the suprachiasmatic and preoptic regions failed to evoke adiposity, even when the paraventricular nuclei were almost completely destroyed The lesions which did produce obesity involved the tuberal or the posterior levels of the hypothalamus, while the most effective lesions lay in or ventrolateral to the ventromedial nuclei, which in the rat are situated at the level of the median eminence just lateral to the third ventricle and ventromedial to the fornices (The swelling or "tuber" visible upon the ventral surface of the rat's diencephalon is caused mainly by the presence of the ventromedial nuclei at that level.) Hetherington and Ranson (1942b) obtained 'markedly obese" rats from bilaterally symmetrical destruction of these nuclei. They found, however, and others have also reported (Brobeck, Tepperman and Long, 1943a) that these nuclei need not be completely destroyed in order to evoke a very remarkable weight gain in the rat, since relatively small lesions placed on the ventrolateral borders of these nuclei and extending to the base of the brain are singularly effective Hetherington and Ranson suggested that axons leave the nucleus along its ventrolateral aspect, where they may be destroyed with relative ease The same fibers can apparently be interrupted at more caudal levels with similar results, since Hetherington and Ranson found that lesions in the posterior part of the lateral hypothalamic area—that is, dorsolateral to the mammillary bodies—were also effective, while Ruch, Patton and Brobeck (1942) obtained obesity in monkeys from still more caudally placed lesions affecting the ventral part of the thalamus, the rostral portion of the mesencephalic tegmentum and the H fields of Forel There is a strong presumption, therefore, that the neurons involved in this disturbance lie in, or at least in the region of, the ventromedial nuclei, that their axons run from the inferior and lateral borders of those nuclei into the region above and lateral to the mammillary

bodies, and from there into the mesencephalic tegmentum (Hetherington, 1944, Brobeck, Tepperman and Long, 1943a, Wheatley, 1944, Graef, Negrin and Page, 1944)

*Energy balance of animals with hypothalamic lesions* The interruption of axons from cells situated in the region identified above profoundly alters the individual's energy balance in such a way as to lead to the retention of a relatively large energy surplus in the form of neutral fat By tradition, this retention has been regarded as a manifestation of some primary disorder of fat metabolism—presumably, as a consequence either of a slowing down of lipid oxidation or of an enhancement of fat synthesis A careful search of the data published from various laboratories, however, yields almost no evidence to justify the opinion that the lesions directly set up any type of metabolic disorder, rather, the data appear to support the interpretation that hypothalamic obesity arises from certain specific deficiencies in the regulation of energy intake and energy output, and that whatever metabolic changes do exist are secondary phenomena arising from the regulatory deficits To attempt to explain the pathogenesis of the obesity, therefore, is to attempt to identify the source of the surplus energy and to determine why the animal spends less energy than it takes in

Basically there are only three possible sources from which this extra energy might be derived They are 1 The food intake of the individual might be increased by the lesions, so that in the presence of an unchanged rate of expenditure, a certain amount of energy is left over for extra storage 2 Food intake might be unchanged, but the amount of energy spent as work might be diminished In an experimental animal this would probably become evident as a reduction in the animal's spontaneous locomotor activity 3 Food and work might be constant, but the energy lost as heat might be decreased This decrease might occur *a*, in that fraction of heat loss which makes up the basal metabolism of the animal, *b*, in the heat associated with muscular exercise, *c*, in the heat liberated in the course of the digestion, assimilation and utilization of food (the so-called "specific dynamic action"), or *d*, in the amount of heat expended by the animal for purposes of temperature regulation In the following discusssion these possibilities will be considered one at a time

1 *Food intake* That animals with appropriate hypothalamic lesions may become obese because they eat large quantities of food appears to have been first suggested by Keller, Hare and D'Amour (1933), who found that certain of the cats and dogs upon which they had performed operations for another experiment showed an "enhanced appetite" At this time no mention was made of obesity, but in two short papers published later, Keller and Noble (1935, 1936) noted that dogs with this "enhanced appetite" became obese, although in many cases the increased food intake as well as the obesity were only of transient duration Keller and his colleagues gave almost no quantitative record of their animal's food intake, nor did they report the rate of weight gain Yet they did make the interesting observation that at least one of the kittens and one of the dogs would drink enormous quantities of milk (but not of water) when they were given no other food Their dog 130 once drank five liters of milk within an

eight-hour period, and before the end of the period was passing undigested milk by rectum. (See also Keller, Noble and Hamilton, 1936) A few years later Krieg (1938) also mentioned "alterations of feeding reactions" in rats with hypothalamic injury, but the nature of the alterations and their possible significance in the etiology of obesity were not further described

Within the past five years, observations similar to those of Keller and his collaborators have been made in several different laboratories upon the rat (Tepperman, Brobeck and Long, 1941, Brobeck, Tepperman and Long, 1943a, Brooks, 1945a, b, c, 1946), the cat (Wheatley, 1944, Ingram, 1945,) the dog (Heinbecker and White, 1944,) and the monkey (Brooks *et al* 1942) When fed *ad libitum*, animals with hypothalamic lesions may eat twice or three times the amount of food eaten by normal animals, as Keller observed, they appear to have an "enhanced appetite" or as stated by Brobeck, Tepperman and Long (1943a), they exhibit hyperphagia[1] Their greatly augmented food intake appears to be a consequence of a highly abnormal attitude toward food—an attitude which is most noticeable within the first few days following the placing of the lesions, but which may persist almost indefinitely and which is intensified by short periods of fasting or by restriction of their food intake They habitually exhibit what appears to be extreme hunger and they may be said not to "eat" their food, but rather to "attack" and to 'devour" it. For example, Wheatley (1944) found that his cats 'ate hurriedly or wolfed their food, while normal cats given the same diet ate slowly, frequently taking several hours to consume their allowance" He wrote also of their "voracious, tigerish appetite," while Ingram (1945) likewise observed that his cats displayed "a high degree of voracity, eating their meals in a fraction of the time taken by most normal cats or by cats with control lesions" When their food intake is limited (as in a paired feeding experiment where the animal with lesions is given only a normal amount of food each day), they may eat their food within a few minutes or an hour, although an equivalent amount will supply a control animal for the full 24 hours (Brobeck, Tepperman and Long, 1943a) A comparable degree of voracity was also observed by Brooks (1945c) in rats used for a paired feeding space-feeding experiment in which small portions of food were delivered to the animals at intervals throughout the day and night Fed in this manner normal rats hoarded their food during the day and ate it at night, while the rats with lesions appeared to be eagerly awaiting the delivery of each morsel which they devoured as soon as it fell into the cage

One of the elements of hypothalamic hyperphagia which makes it such a striking, impressive phenomenon is the abruptness of its onset In the rat, for example, where the hypothalamic operation is usually performed with the rat under the influence of a short-acting barbiturate, ravenous eating may appear

[1] The word 'hyperphagia" was chosen because it does not have the subjective psychological connotations of the terms "hunger" appetite satiety' and 'bulimia' and because the word 'polyphagia' which might have been adopted implies 'omnivorousness, craving for all kinds of food (Dorland) Hyperphagia is taken to mean simply increased eating

even before the effects of the anesthetic are completely gone, and the food intake of the first post-operative day may be three times the normal daily intake In many instances the animal eats more during the first 24 hours than it ever eats during a similar period thereafter, it is not uncommon to find that on this first day the food intake jumps to a high peak and that it subsequently declines gradually as the animal becomes more and more obese Expressed in terms of the dynamic and static phases of the obesity, the food intake may be greatest at first and less as the animal approaches and enters the static phase There are certain animals, however, which never enter a true static phase because they continue to overeat and to gain weight almost indefinitely These are the animals in which the the greatest deficit appears to be present, and it is evident, therefore, that the deficit is not an all or nothing phenomenon, that some operations are more successful than others, and that there is a considerable amount of variation in the degree of the hyperphagia exhibited by different animals

At least two partially successful attempts have been made to ascertain the significance of the tendency shown by many rats to return to a normal food intake as they become obese There may be a certain amount of recovery from the effects of the operation, but the following experiment indicates that this is not the only explanation of the gradual decrease in food intake A rat will regain its hyperphagia if it is first fasted long enough to allow it to return to a normal body weight To perform this experiment the animal is allowed to grow obese, it is then fasted for 20-35 days, depending upon just how obese it has become, and finally, it is given food *ad libitum* once more It will then become obese a second time (Brobeck, Tepperman and Long, 1943a, Brooks, 1945c)

During the period of extreme hyperphagia immediately following the operation, a rat may gain as much as 42 grams in 24 hours, where the normal rate of gain is only 0 5–1 0 gram per day (Brobeck, unpublished) Most of this immediate gain must represent the food and water with which the gastrointestinal tract is distended, yet the rat may continue to outgain its control by a ratio of more than 5 1 for several weeks or more, and in this case the weight gain must represent an actual increase in tissue constituents, i e , in neutral fat Brobeck, Tepperman and Long (1943a) found that there was a significant correlation between food intake and weight gain in their rats, since the more the animals ate, the more weight they gained, and this observation led the authors to conclude that the hyperphagia was primarily responsible for the development of this type of obesity

Whether hyperphagia is solely responsible for the abnormal weight gain, however, is a debated topic (Hetherington and Ranson, 1942c, Brobeck, Tepperman and Long, 1943a, Brooks, 1946, Brooks and Marine, 1946, Ingram, 1945) To attempt to answer the question, animals with lesions have been pair-fed with normal controls by giving the operated animal the quantity of food eaten by the control on the previous day (or during the previous week) The energy intake is thereby held to a normal level at which the weight gain of the two animals may be directly compared The results apppear to show that while hyperphagia is the most important factor and while in many animals it may be the only factor, there are other factors which have smaller though appreciable effects upon weight gain The experience in Long's laboratory may be taken as a typical one

Twelve paired feeding experiments were reported, in nine of them the rat with lesions gained at the same rate as the control with which it was paired until the time *ad libitum* feeding was instituted, whereupon the former animal almost immediately began to become obese because it increased its food intake Here the hyperphagia appeared to be solely responsible for the obesity In the other three pairs of the series, the rat with lesions gained a little more rapidly than the control during the paired feeding, yet when *ad libitum* feeding was permitted the former animal underwent an abrupt rise both in food intake and in weight gain. Here the hyperphagia, although clearly the most important, was not the only factor responsible for the excess gain To discover the nature of the other factors it is necessary to enquire further into the behavior of the animal with hypothalamic lesions

One of the most popular hypotheses to account for the obesity of human patients who are said to eat only a normal quantity of food is the idea that their food is unusually well digested and absorbed from the gastrointestinal tract. Thus their total energy intake might be greater than that of normal individuals even though the total amount of food actually eaten by the two groups might be identical Unfortunately for the proponents of this view, there appears to be no experimental evidence to support the theory, such measurements as have been made indicate that abnormal weight gain in general cannot be accounted for on this basis (Evans, 1942, Newburgh, 1944) In the case of animals with hypothalamic lesions, however, intestinal digestion and absorption have not been measured, and whether or not a greater proportion of the foodstuffs is absorbed is unknown. Presumably this is not an important source of extra energy, since the obesity of animals upon *ad libitum* feeding can be attributed to their hyperphagia, and since the abnormal weight gain of pair fed animals appears to be the result of a reduction in locomotor activity or in total heat production or in both of these factors (see below)

2 *Spontaneous locomotor activity* Muscular exercise is the second important variable factor in physiological energy exchange If all other factors remained constant, the energy surplus leading to hypothalamic obesity theoretically might arise solely from a reduction in the animal's motor output. This possibility may be investigated in a laboratory animal such as the rat by means of one or another type of "activity" cage The type most widely used consists of a freely turning circular cage attached to a "counter" which records the number of revolutions through which the cage turns as the animal walks or runs inside it With activity cages of this design Hetherington and Ranson (1942c) discovered that the production of hypothalamic lesions brings about a brief period of extreme hyperactivity which usually begins even before the animal has completely recovered from the anesthetic used for the operation, and which persists for several hours.[4] Then the hyperactivity gradually ceases, to be replaced by a period of inactivity which appears to be a permanent deficit, since it persists for

[4] Brobeck Tepperman and Long (1943a) found that when the rats were given food during the period of hyperactivity many of them would immediately stop moving around the cage in order to attack the food which they devoured in large quantities The ravenous eating appeared to be a substitute for the 'frenzied' activity which it replaced, or vice versa

several months or more with only minor fluctuations Their observations were confirmed in the rat by Brooks (1946) and by Brobeck (unpublished), and in a general way in the cat by Ingram (1945) Hetherington and Ranson appear to have recorded in quantitative terms a phenomenon that had already been qualitatively described as a tendency toward inactivity and somnolence in animals with large lesions of the posterior hypothalamus (Ranson and Magoun, 1939)

There is general agreement, therefore, regarding the effect of hypothalamic lesions upon locomotor activity, but there is a division of opinion over the significance of this inactivity as a cause of obesity In the opinion of Hetherington and Ranson (1942c), the inactivity was a primary source of the surplus energy stored as fat by their animals, and the food intake was a factor of minor importance Other workers, however, favor the view that the hyperphagia is the basic and most important source of energy imbalance, especially when the animal is fed *ad libitum*, and that the inactivity is, according to Brooks (1946), "at most, merely a contributory cause of obesity," the effect of which is important only under certain conditions (Brobeck, Tepperman and Long, 1943a, Ingram, 1945) For example, when the food intake is arbitrarily limited in amount as in paired feeding, an inactive animal may store as fat the energy spent in locomotion by its more active control animal, and consequently, the former may slightly outgain the latter (Brobeck, 1945) This may be one explanation for the results of the paired feeding experiments described above where a relatively small number of animals were found to gain weight more rapidly than the controls with which they were paired But the following observations suggest that this mechanism is ordinarily not an important cause of obesity

*a* Inactivity by itself does not always lead to obesity (Brooks, 1946) Thus, all of the ten male rats studied by Hetherington and Ranson (1942c) were inactive, but only six became obese, and four of these six ate more food than their controls If inactivity were necessarily related to obesity, all ten animals should have gained extra weight Similarly, Brobeck (unpublished) found that locomotor inactivity of varying degrees occurred in all of twelve male and twelve female rats with hypothalamic lesions, yet none of the animals gained extra weight so long as they were given only the amount of food given to their controls (The controls had been trained to eat their food as the rats with lesions ate, in a short period of time) Yet when they were given food without limitation, the majority of the animals with lesions almost immediately began to become obese *b* Although the obese rat does not walk or run much in an activity cage, its total energy expenditure for muscular exercise may be elevated above the normal level because it must exert more force in moving the fat-laden members of its body during respiration, eating, drinking, etc Brooks (1946) was led to this conclusion by the results of an experiment performed upon rats housed in cages where motor activity was recorded upon a kymograph through a system connected with rubber tambours upon which the cage was suspended His apparatus recorded all types of activity great enough to disturb the equilibrium of the system With it Brooks observed that the periods of hyperactivity and inactivity described by Hetherington and Ranson (1942c) were followed by a

period characterized by bouts of hyperactivity that finally became almost continuous as the animal became obese He interpreted this to mean that the tambours were being compressed by movements too slight to have been recorded when the animal was of normal size and weight, and he suggested that "the energy requirement for activity obviously increased as the animals became obese" His interpretation agrees well with Newburgh's (1944) conclusions regarding the energy expenditure of the obese human patient during muscular exercise.

3 *Heat production* Finally, the animal with hypothalamic lesions might divert into its storage mechanisms energy normally dissipated as heat with the result that the obesity would depend upon a reduction in the total heat production. Food would then be utilized at a higher "efficiency," i e. , the proportion of food energy expended as heat would be depressed and relatively more energy would be available for storage. The body temperature would tend to be proportionately below normal unless a normal temperature could be maintained through the activity of the mechanisms of heat conservation, such as pilomotor vasomotor and postural changes which are ordinarily not active at the usual laboratory temperatures. This possibility is an interesting one because the hypothalamus is known to be largely responsible for the regulation of body temperature, even though the rate of heat production has not been shown to be under direct hypothalamic control (Clark, Magoun and Ranson, 1939) However attractive the hypothesis may be, it cannot account for the obesity of animals fed *ad libitum* because their heat production rises as they gain weight Estimated from their total oxygen consumption under fasting, resting conditions, their total heat production may become twice as great as it was before the hypothalamic operation (Bruhn and Keller, 1941, Brobeck, Tepperman and Long, 1943a, Brooks and Marine, 1946) Yet the animal may continue to gain weight, this can only mean that the obesity originates primarily from some source other than the conservation of energy which a normal animal spends as heat. The foregoing discussion will make it apparent that the energy surplus is for the most part a consequence of the hyperphagia

Nevertheless, there is evidence which suggests that rats with hypothalamic lesions may undergo a diminution in total heat production within the first few days after the production of the lesions Since this diminution shortly becomes obliterated by the rise associated with the developing obesity, it can be detected only by testing the animals before their weight has changed appreciably, or by preventing abnormal weight gain by limiting their food intake (Brooks and Marine, 1946) Quantitatively the amount of energy made available for extra storage by the reduced heat production is almost insignificant in contrast to the huge excess ordinarily taken in as food Yet the data of Brooks and Marine indicate that the rate of heat production may be an important factor in precise studies of energy exchange, and may well explain the ability of certain animals to outgain their controls during paired feeding (Hetherington and Ranson, 1942c, Brobeck, Tepperman and Long, 1943a, Ingram, 1945)

How the hypothalamic damage alters the basal heat production is unknown.

The observation that the change comes on gradually over a period of days (Brooks, 1945c) suggests that it is a secondary phenomenon, dependent upon some other more direct effect of the lesions It may prove to be a result of the animal's disturbed feeding habits Thus, Brobeck, Tepperman and Long (1943a) had previously suggested that the ability of a certain few rats with hypothalamic lesions to gain extra weight during paired feeding was a consequence of their abnormal feeding habits, or specifically, of the fact that the hyperphagic animals ate a normal 24-hour portion of food within one or two hours If this interpretation of the phenomenon is correct, the unusual weight gain should disappear if the animals could be induced to eat normally Unfortunately, it has not been possible to devise a method for restoring normal eating habits in these rats Brooks (1945c) was able to approach this goal, however, by conducting paired feeding experiments in which the operated animals could not eat their daily portion all at once because the food was supplied in small amounts at 4-hour intervals throughout the day and night On this regimen some of the operated rats still outgained their controls, but a rather considerable difference between the eating habits of the two groups of rats persisted throughout the experiment, inasmuch as the operated rats showed strongly developed anticipatory behavior when the food was about to be delivered, and promptly consumed each pellet. By contrast, the normal rats let their food accumulate until night time, when they ate it according to their usual schedule Brooks' observations suggest, therefore, that the source of the positive energy balance in the pair-fed rat with lesions is not to be found in the habit of eating a large amount of food all at once, but his experiment does not rule out the possibility that the difference in weight gain is derived in some way from the dissimilar eating habits of the two groups of animals This problem has also been approached indirectly by training normal rats to eat as quickly as the operated, pair-fed rats do, when the controls are so trained, they gain at the same rate as the operated rats with which they are pair fed (Brooks, 1945c, Brobeck, unpublished) It seems probable, therefore, that the time of eating is an important consideration in the study of energy balance, or that the manner in which food is utilized depends to a certain extent upon the eating habits of the animal More definite conclusions must await further evidence

*Intermediary metabolism of foodstuffs in animals with hypothalamic lesions* From data now available the intermediary metabolism of foodstuffs in the animal with hypothalamic lesions cannot be described with any degree of confidence Here may be a potentially profitable field for research Data hitherto reported appear to point to two general conclusions 1 That the abnormalities which may be present have to do with the rates at which chemical reactions occur, since the reactions *per se* are not unusual although the rate at which they proceed may be quite different from those of the normal individual 2 The differences appear to be the result of the animals' abnormal eating habits, or, specifically, of their tendency to hyperphagia

There appears to be nothing remarkable about the chemical structure of the accumulated fat Hetherington and Weil (1940), who made a limited analysis

of the carcasses of two obese rats with purely hypothalamic lesions (as well as of two rats with combined hypophysial and hypothalamic lesions), found that the surplus body mass was composed almost entirely of fat which had normal iodine and saponification numbers. They also determined the total and the relative quantities of phosphorus, calcium and iron in the whole carcass and in the acetone and in the alcohol fractions, and discovered that the absolute quantity of each of the three elements was the same as in normal rats. Expressed in percentage of the total weight of the carcass, however, there was an apparent depletion of phosphorus and calcium, and possibly also of iron. Hetherington and Weil interpreted the percentage changes to be indicative of an 'obviously disordered fat metabolism". Nevertheless another interpretation appears to be possible, namely, that except for the surplus fat, the composition of the carcasses was normal, since the absolute quantities of phosphorus, calcium and iron were within normal limits and since these elements are normally present mainly in the non-adipose tissue of the body. The obese carcass is probably composed of two fractions, viz., 1, the non-adipose tissues (containing most of the P, Ca and Fe) which are of normal size and composition, and 2, the adipose tissue which has a normal composition but a tremendously increased total mass. The latter tends to "dilute" the former when the concentration of any element present mainly in the non adipose tissues is expressed as a percentage of the total body mass. The "depletion" observed by Hetherington and Weil, therefore, may be only an apparent one derived from their method of expressing their results, and their data may or may not mean that the intermediary metabolism of fat is "disordered".

There has been a tendency to suppose that the animals are unable to metabolize the fat which accumulates in such great quantities, but the available experimental evidence indicates that this supposition is unjustified. Obese rats and monkeys can and do utilize tissue fat when their food supply is inadequate (Brooks, Lambert and Bard, 1942, Brobeck, Tepperman and Long, 1943a). They may be fasted for long periods of time (as long as 35 days, Brobeck, unpublished) while they use up their body fat and lose weight at a rate which suggests that fat is furnishing most of the energy for their metabolic processes. Upon refeeding, obesity may reappear. Further evidence that fat is oxidized without difficulty is to be found in the observation that the respiratory quotient of the fasting obese rat is within normal limits, between 0.700 and 0.760, well within the range associated with fatty acid utilization (Tepperman et al., 1941).

The intermediary metabolism of dietary carbohydrate, however, is known to be altered in animals with hypothalamic hyperphagia, since Tepperman et al. (1943) obtained evidence that carbohydrate is transformed to fat at a tremendously accelerated rate. The evidence was obtained by comparing the respiratory quotient of normal and of operated rats in the fasted state, in the fed state, and during the absorption of glucose solution. Fasted, the R.Q. was the same in both groups of rats (as noted above), which indicates that both were utilizing for energy and beat the same substrate, mainly fatty acids. When normal rats were fed or given glucose the R.Q. rose a little undoubtedly because a

of the carbohydrate was oxidized (R Q 1 000) and some was converted to fat (R Q above 1 000) Under these same conditions, on the other hand, the R Q of the hyperphagic rats was found to be exceptionally high, well above unity and within the range to be expected only if most of the absorbed carbohydrate was being converted to fat Tepperman et al (1943) concluded that the R Q change was not a direct result of the hypothalamic damage because they found that just after the operation, rats disposed of glucose in the same way as did the controls, according to Brooks (1945b) the adaptation gradually appears within the first postoperative week, as might be expected if the phenomenon arose from the animals' hyperphagia Tepperman also discovered that normal rats could be induced similarly to convert glucose to fat preferentially if they were trained to eat their 24-hour portion of food within a short period of time In some way or other the training process accelerates the rate of the conversion reactions, possibly because the adipose tissue constitutes the only depot large enough to hold the amount of material which must be stored when the animal can eat only once a day In the opinion of Tepperman et al "The studies on unoperated animals show that periodic overfeeding of carbohydrate results in an increased tendency to convert carbohydrate to fatty acids, the experiments on *ad libitum* fed rats with hypothalamic lesions, some of which ate from two to three times as much as did their controls, demonstrate a similar response to stress on the part of the conversion mechanisms Thus, the tissues of the overnourished animal may be presented with a metabolic mixture of carbohydrate and fat that bears little resemblance to the proportionate representation of those substances in the normal diet Schoenheimer and Rittenberg (1935) showed that even the normal animal in caloric balance converts some ingested carbohydrate to fat, and that the fat depots are by no means inert storage places from which an energy source is mobilized only during fasting The present studies suggest that, in the overnourished animal, there may be a marked accentuation of the processes described by these authors " In support of this conclusion, Dickerson, Tepperman and Long (1943) found that a high R Q in the presence of glucose was manifest also in liver slices taken from trained animals, and that the liver appeared to be playing an important rôle in the carbohydrate-fat conversion process The reaction, itself, is not abnormal, its unusual character in rats with hypothalamic lesions and in trained normal rats lies in the unusually rapid rate at which the conversion takes place and in the large quantity of carbohydrate being disposed of in this way

Tepperman and his colleagues discovered that insulin plays an essential part in the reactions which bring about the rise in respiratory quotient observed in their animals, and in the light of Drury's (1941) evidence that insulin promotes fat storage, they suggested that relatively large amounts of insulin might be needed by animals synthesizing large quantities of fat from carbohydrate The well known association between human obesity and diabetes mellitus (Evans, 1942) has been advanced as an argument in favor of this hypothesis by Brobeck, Tepperman and Long (1943b), who showed that hyperphagia does increase the rat's insulin requirement, since the production of appropriate hypothalamic

lesions with resulting hyperphagia provoked a striking glycosuria in previously non-diabetic, partially depancreatized rats. When the food intake was restricted to the preoperative level the glycosuria decreased in intensity and eventually disappeared. The remnant of islet tissue present in a partially depancreatized rat evidently liberates just enough insulin to provide for the metabolism of normal amounts of food, and when the food intake is so greatly increased under the influence of the diencephalic injury, insulin can no longer be elaborated rapidly enough for disposal of all of the carbohydrate, hyperglycemia and glycosuria then develop. The rat whose pancreas has not been surgically reduced in size is better able to take care of large quantities of food, but even here a latent insulin deficiency may be demonstrated by suitable tests. For instance, in the presence of long standing obesity the rat may show impaired glucose tolerance as well as occasional, spontaneous glycosuria (Brobeck, Tepperman and Long, 1943b). In this connection it is appropriate to recall the report of Ranson, Fisher and Ingram (1938) where the authors described an obese monkey with diabetes mellitus, although they were not convinced that either the obesity or the diabetes arose from the hypothalamic damage they had inflicted. More recently, Ruch and Patton (1946) observed another obese monkey with unmistakable diabetic tendencies if not frank diabetes mellitus. The evidence is reasonably convincing, therefore, that the animal with hypothalamic lesions may undergo changes in the intermediary metabolism of carbohydrate which are the result of habitual overfeeding.

*Origin of the hyperphagia.* To recapitulate. Experimental data have shown that the obesity of animals with hypothalamic lesions arises mainly from a marked increase in food consumption. This increase may be accompanied by a decrease in locomotor activity and by a transitory decrease of basal heat production, neither of which plays any constant, essential part in the genesis of obesity under ordinary laboratory conditions. Only when the food intake is arbitrarily held at a normal level is their relatively small influence on total energy exchange detectable. When the food intake is not restricted extra food eaten by the animal constitutes a relatively large energy load which the tissues dispose of by storing some of it and by oxidizing the excess to carbon dioxide and water. Hence, the obesity and the elevated total metabolism of the obese animal. Expressed in this way as a problem in energy balance, the situation appears to be fairly clear—the more the animal eats the more obese it becomes. But expressed in physiological terms as a problem in the regulation of energy exchange, the situation is still obscure. The question "Why does the animal with hypothalamic lesions eat so much food?" immediately raises the questions, "Why does the animal eat food?" What determines how much it will eat? What changes in the internal environment set the animal to eating, and what changes are produced by satiety?

Although the evidence appears to be incomplete, hyperphagia may be tentatively regarded as a consequence of a particular disturbance of the mechanisms which regulate energy exchange. Normally the rate of work output and heat production are controlled in such a manner that

an equilibrium between energy intake and energy output, as a result, the amount of energy stored as fat remains almost constant from day to day and from year to year, even though the organism must submit to variations in diet, in work and in environmental temperature The precision with which this equilibrium is maintained suggests that there must be some quantitative regulation and integration of the variable factors upon which the equilibrium rests, i e , of food intake, work output and heat loss One can hardly fail to be impressed by the profound disturbances in the regulation of each of these factors which have been observed to follow appropriate hypothalamic injury Obesity is one such disturbance, the result mainly of a failure of regulation of energy intake, the others include the deficiencies of temperature regulation described by Clark, Magoun and Ranson (1939) and by Ranson (1940), and the locomotor inactivity reported by Hetherington and Ranson (1942c, see above ) That lesions of the hypothalamus induce these highly typical deficits seems to indicate that the hypothalamus normally participates in the maintenance of the overall energy equilibrium, and that the control of food intake, work output and body temperature may be correlated and integrated within this portion of the diencephalon

## REFERENCES


Aschner, B Pflüger's Arch **146** 1, 1912

Babinski, M J Rev neurol **8** 531, 1900

Bailey, P and F Bremer Endocrinology **5** 761, 1921a Arch int Med **28** 773, 1921b

Bard, P Res Publ Ass nerv ment Dis **20** 551, 1940

Bell, W B Quart J exper Physiol **11** 77, 1917

Biggart, J H and G L Alexander J Path and Bact **48** 405, 1939

Brobeck, J R Am J Physiol **143** 1, 1945

Brobeck, J R , J Tepperman and C N H Long Yale J Biol Med **15** 831, 1943a Yale J Biol Med **15** 893, 1943b

Brooks, C McC Fed Proc **4** 9, 1945a Fed Proc **4** 9, 1945b Personal communication, 1945c Fed Proc **5** 12, 1946

Brooks, C McC , E F Lambert and P Bard Fed Proc **1** 11, 1942

Brooks, C McC and D N Marine Fed Proc **5** 12, 1946

Bruch, H Am J Dis Child **58** 1282, 1939

Bruhn, J M and A D Keller Am J Physiol **133** 229, 1941

Camus, J and G Roussy C R Soc Biol Paris **75** 483, 1913 Rev neurol **36** 1201, 1920 Rev neurol **38** 622, 1922

Clark, G Science **90** 92, 1939

Clark, G , H W Magoun and S W Ranson J Neurophysiol **2** 61, 1939

Crooke, A C and J R Gilmour J Path and Bact **47** 525, 1938

Crowe, S J , H Cushing and J Homans Johns Hopkins Hosp Bull **21** 127, 1910

Dey, F L Endocrinology **33** 75, 1943

Dickerson, V C., J Tepperman and C N H Long Yale J Biol Med **15** 875, 1943

Dott, N M Quart J exper Physiol **13** 241, 1923

Drury, D R Am J Physiol **131** 536, 1941

Erdheim, J S B Akad Wiss Wien, Abt III, **113** 537, 1904

Evans, F A In G G Duncan Diseases of metabolism, Chapter 10, pp 513–691 Philadelphia, Saunders, 1942

Fröhlich, A Wien klin Rdsch **15** 882, 906, 1901 Res Publ Ass nerv ment Dis **20** xvi, 1940 Am J Dis Child **58**. 1282, 1939 Discussion following paper by P Bailey Res Publ Ass nerv ment Dis **20** 713, 1940

GRAEF, L., J NEGRIN, JR. AND I H PAGE Am J Pathol 20: 823, 1944
GRAFE, E AND E GRÜNTHAL Klin Wchnschr 8 1013 1929
HEINBECKER, P., H L WHITE AND D ROLF Am J Physiol 141: 549, 1944
HETHERINGTON, A W Endocrinology 26: 264 1940 Am J Physiol 133 326 1941 Am J Physiol 140 89, 1943 J Comp Neurol 80: 33 1944
HETHERINGTON, A W AND S W RANSON Proc Soc exper Biol, N Y 41 465 1939 Anat. Rec 78 149 1940 Endocrinology 31: 30 1942a J Comp Neurol. 76 475, 1942b Am J Physiol 136 609 1942c
HETHERINGTON A W AND A WEIL. Endocrinology 26 723 1940
INGRAM, W R. Personal communication 1945
KELLER A. D W K. HARE AND M C D'AMOUR Proc Soc Exper Biol, N Y 30: 772 1933
KELLER, A D AND W NOBLE Am J Physiol 113 79 1935 Am J Physiol 116: 90, 1936
KELLER, A. D, W NOBLE AND J W HAMILTON JR. Am J Physiol 117 467, 1936
KRIEG, W J S Anat. Rec 70 suppl 3: 48, 1938
MCHENRY E. W AND J M PATTERSON Physiol Rev 24 128 1944
MEANS, J H Arch int Med 17: 704 1916
MOHR. Wchnschr ges Heilk pp 565–571, 1840
NEWBURGH, L H. Physiol Rev 24 18 1944
RANSON, S W Res Publ Ass nerv ment Dis 20: 342 1940
RANSON, S W, C FISHER AND W R INGRAM Endocrinology 23 175 1938.
RANSON, S W AND H W MAGOUN Ergebn Physiol 41 56 1939
RONY, H R. Obesity and leanness Philadelphia, Lea & Febiger, 1940
RUCH, T C., M BLUM AND J BROBECK Am J Physiol 133: P433 1941
RUCH, T C AND H D PATTON Personal communication 1945
RUCH, T C, H D PATTON AND J R BROBECK Fed Proc 1: 76 1942
SCHOENHEIMER, R. AND D RITTENBERG J Biol Chem 111 175 1935
SMITH P E J A M A 88 158 1927 Am J Anat 45 205 1930
TEPPERMAN, J J R BROBECK AND C N H LONG Am J Physiol 133: 468, 1941 Yale J Biol Med 15: 855, 1943
WHEATLEY M D Arch Neurol Psychiat Chicago 52: 296 1944

# CARBONIC ANHYDRASE IN TISSUES OTHER THAN BLOOD

HORACE W DAVENPORT

*Department of Physiology, University of Utah School of Medicine, Salt Lake City, Utah*

The reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ proceeds at a finite rate in the absence of any catalyst, and the reaction is catalyzed by many anions and inorganic compounds of non-enzymatic nature which are present in tissues (84) A reaction velocity equation which takes into account the non-enzymatic catalysis has been described by Roughton, and the numerical values of the several constants occurring in the equation are known (82)

The reaction is also catalyzed by the enzyme, carbonic anhydrase The enzyme was originally sought for and discovered in the erythrocytes because it was shown that the rate of the reaction in the absence of an enzyme is not fast enough to account for the known rate of liberation of carbon dioxide in the lungs (80, 81) Without carbonic anhydrase the release of the amount of carbon dioxide liberated from the blood during one passage through the lungs would go to within 90 per cent of equilibrium in about 100 seconds Since erythrocytes spend less than one second in the lung capillaries (83), enzymatic catalysis of the reaction is required

When the function of carbonic anhydrase in tissues other than blood is being considered the fact must always be kept in mind that the rate of the reaction uncatalyzed by carbonic anhydrase *does have a finite velocity* If the rate of reaction required by the metabolic activity of the tissue is not limited by blood flow or other mechanical factors the rate of the reaction uncatalyzed by carbonic anhydrase may be fast enough Therefore, it is necessary to demonstrate for each tissue in which the enzyme is present that enzymatic catalysis of the reaction is actually required The demonstration of the presence of the enzyme in a particular tissue is not proof that the enzyme has an indispensable function there

*Distribution in tissues of warm-blooded animals* It is difficult to compare the reported values for the concentration of the enzyme in various tissues, because the unit in which the activity of the enzyme is to be expressed has not been agreed upon Important as the exact definition of the unit may be for chemical work on the enzyme (58, 60, 86, 87, 88, 89), it is not important for physiological work. There is at least one thousand times as much enzyme present in the erythrocytes as is needed to furnish the required catalysis of the dehydration of carbonic acid in the lungs (81, 82) In most tissues where the enzyme is definitely known to be present its concentration is probably no *less than one-tenth* and certainly no less than one-thousandth that of the erythrocytes It is probable that there is considerable excess of carbonic anhydrase in tissues other than blood Small differences in concentration between tissues or in various parts of a tissue cannot be assigned any functional significance, especially when a cellular locus of the enzyme has not been determined

The enzyme occurs in very high concentrations in erythrocytes (72, 92)

Pure preparations of the enzyme have only 150 times as much activity as the erythrocytes when the two are compared on the basis of dry weight (58) The enzyme is highly concentrated in the gastric mucosa (34), and it may have a higher concentration in the parietal cells of the gastric mucosa than in the erythrocytes (23, 24) High concentrations are also found in the kidney cortex (36), the proventriculus and oviducts of fowls (22), and in the lens and retina (8, 9)

The enzyme occurs in lower but still significant concentrations in brain (1,-2,3,4,5,6), liver (1,48), testis (48), pancreas (48), spleen (48), red muscle (48), the mucosa of the duodenum, ileum and jejunum (33), the uterus and magnum of fowls (22), and saliva (79) The enzyme occurs only in traces if at all in striated muscle (48), heart muscle (48), the choroid and retina of the eye (7), the small intestine of fowls (22), and the spinal cord of the hog (2), and the adrenal glands (1)

The enzyme is definitely absent from normal plasma (72), normal urine (72), milk (72) and the shells, whites and yolks of unfertilized hens' eggs (22) It is also absent from the sclera of the eye (7), the gallbladder (48), skin (48), lungs (48) and thymus (33)

The presence of the enzyme in several tissues has been disputed Although the enzyme is undoubtedly present in the gastric mucosa (34) and in the kidney cortex (36) this has been denied (15) The concentration of the enzyme in mammalian peripheral nerve has been reported as "trace" (48) The reviewer finds the concentration to be less than 0 005 of his units per milligram wet weight in cat peroneal nerve perfused completely free of blood (33)

There are large but probably insignificant species variations in the concentration of the enzyme

*Distribution in lower animals* Carbonic anhydrase is present in frog erythrocytes (70), kidneys (33), gastric mucosa (33) and the lens of the eye (7), though in much lower concentration than in the corresponding mammalian tissues There is no carbonic anhydrase in frog sciatic nerve perfused free of blood (33) The enzyme is present in a very large number of fish and invertebrate tissues, among them the pseudobranchial glands, spleen, gills, stomach, pyloric appendices and air bladders of many fish (96), and the gills of many crabs, molluscs and echinoderms It is impossible to summarize here the invertebrate distribution, and the reader is referred to the original papers for details (18,41,42,45,46 62,65,74,96)

*Origin, precursors and fate* Since zinc is a constituent of carbonic anhydrase (57) an adequate dietary supply of the element must be necessary for the formation of the enzyme However, there is no reduction in either the enzyme or the zinc content of the blood of rats showing severe symptoms of acute zinc deficiency (55) By no means all the zinc in the tissues is contained in the carbonic anhydrase (67, 93) It has not been shown that other components of the diet are necessary for carbonic anhydrase formation. An attack on the problem of carbonic anhydrase formation using the methods by which Whipple and his colleagues have studied the formation of hemoglobin and plasma protein would doubtless yield important information.

The site of formation of the enzyme is unknown The carbonic anhydrase concentration of the erythrocytes of fetal goats (72) and of premature infants (98) is low, and the concentration arises abruptly near term The appearance of the enzyme in the blood should be compared with its appearance in other tissues in mammals In the developing chick the enzyme appears in the lens of the eye on the fourth day but not in the blood until the twelfth day (47), suggesting that the enzyme may be formed independently in each tissue

The enzyme present in tissues may differ from that in the erythrocytes (33) Between 20 and 40 per cent of the enzyme in aqueous extracts of gastric mucosa or kidney is completely uninhibitable by the specific protein inhibitor discovered by Booth (17) By precipitation with ammonium or sodium sulfate a fraction can be prepared none of whose carbonic anhydrase activity can be inhibited by the protein inhibitor and which probably has a higher molecular weight than the enzyme from the erythrocytes When the uninhibitable fraction is allowed to stand in buffer solutions it slowly becomes inhibitable These observations suggest that the enzyme as it exists in tissue is closely associated with another protein, and they may explain the difficulty which Keilin and Mann (58) experienced in their attempt to purify the enzyme from the gastric mucosa The whole question of the origin and nature of the enzyme in tissues should be investigated

There is no carbonic anhydrase present in plasma as the result of the normal breakdown of erythrocytes (72) Even if some of the enzyme were liberated by the destruction of erythrocytes there would be no enzyme activity in the plasma of those animals whose plasma contains the specific protein inhibitor When large amounts of laked erythrocytes are injected intravenously into dogs, the carbonic anhydrase present in the plasma disappears at about the same rate as the hemoglobin (30) No carbonic anhydrase appears in normal urine, and none has been found in the urine of patients suffering from hemolytic diseases (64) However, the enzyme, whose molecular weight is 30,000 (38, 75), can pass through the glomerular membrane When large amounts are injected intravenously into dogs previously given sodium bicarbonate the enzyme appears in the urine (30), but only a very small percentage of the enzyme disappearing from the blood can be accounted for by that appearing in the urine The clearance of the enzyme is always less than four per cent of the simultaneous creatinine clearance, and it decreases very sharply with decreasing plasma concentration This probably does not mean that the enzyme is reabsorbed by the tubules to any significant extent but rather that the compound which the enzyme forms with the protein inhibitor (a pseudo-globulin) present in dog plasma has a molecular weight too high to pass through the glomerular membrane

Variations in the enzyme content of the blood in disease throw no light on its origin or fate A slight fall has been reported in tuberculosis and a small rise in diabetes (94, 95) No change occurs in rats with implanted tumors (66) The maximum deviations from normal reported in a large variety of blood disease is only about five-fold (53, 64) Considering the thousand-fold excess of enzyme in erythrocytes the variations recorded are not significant, nor are

those found in brains of psychotic patients at autopsies (4, 5) No observations have been made on the possible absence of carbonic anhydrase from the gastric mucosa of patients with pernicious anemia, nor have variations in the enzyme content of other tissues been studied.

*Inhibition of carbonic anhydrase* Inhibitors of carbonic anhydrase have been used in most of the physiological work on the functions of the enzyme in tissues other than blood The enzyme is inhibited by protein poisons, heavy metals and cyanide (72) and other substances, but none is a useful physiological tool The first inhibitor to be used in animal experiments was thiocyanate (25) which inhibits gastric acid secretion as well as carbonic anhydrase On the basis of the explicit assumption that the rate at which carbonic acid is formed in the acid secreting system is proportional to the fraction of uninhibited carbonic anhydrase present it was calculated that the rate of acid secretion is directly proportional to the rate of carbonic acid formation The facts and the conclusion are probably correct, but the reasoning is wrong The assumption, although apparently reasonable, is false, and since it or similar assumptions underlie much of the speculation on the function of the enzyme it deserves detailed analysis

The reaction by which thiocyanate and sulfonamide drugs having a free sulfonamide group (69) inhibit the enzyme can be expressed as $E + I \rightleftharpoons EI$ (31) $E$ is the free, catalytically active enzyme, I the inhibitor, and EI a catalytically inactive compound of enzyme and inhibitor The reaction is almost instantaneous and reversible The mass action expression for the reaction is $(E)\ (I - EI)/(EI) = k$ Since the molar concentration of inhibitor so far used is at least one hundred times the molar enzyme concentration, the equation can be reduced to $(E)(I)/(EI) = k$ The constant for any inhibitor can be determined by measurement of the fractional activity of the enzyme at various inhibitor concentrations, and it is independent of the enzyme units and the total concentration of the enzyme It is apparently independent of the substrate concentration for the reason that the affinity of the enzyme for its substrate is very much greater than its affinity for any known inhibitor The constant is also independent of the source of the enzyme The constant at 38°C and pH 6 8 is about $3.5 \times 10^{-3}$ for thiocyanate (25), about $7\ 4 \times 10^{-4}$ for sulfanilamide (31) and about $1\ 5 \times 10^{-7}$ for thiophene-2-sulfonamide (31)

If the inhibitor concentration is known the fraction of the enzyme inhibited can be calculated It is difficult to determine in cells the relation between the fractional inhibition of the enzyme and the fractional inhibition of the catalyzed reaction. The reactions are so fast that they cannot be studied under physiological conditions of temperature and substrate concentrations by ordinary methods To obtain an approximate description of the effect of inhibitors upon the catalyzed reaction the rate of carbon dioxide uptake by blood has been studied at 0 C and 15°C with large substrate changes. The results obtained by Keilin and Mann (59) with their ingenious spectrometric method and with equally large substrate changes are similar If less than 97 per cent of the enzyme is inhibited the catalyzed reaction is entirely uninhibited, and in order

to reduce the rate of the catalyzed reaction by 50 per cent more than 99 5 per cent of the enzyme must be inhibited To reduce the catalyzed rate by 90 per cent more than 99 8 per cent of the enzyme must be inhibited These results agree with Roughton's calculation that a great excess of the enzyme exists in the erythrocytes

The important conclusions are that thiocyanate at a concentration of 0 05 M or less cannot be expected to inhibit the catalyzed reaction at all, and that in order to attain 90 per cent inhibition of the catalyzed reaction the sulfanilamide concentration must be about 0 0073 M (125 mgm per cent) Thiophene-2-sulfonamide will probably produce 90 per cent inhibition at a concentration of 0.0014 M (25 mgm per cent)

The determination of the percentage inhibition of the enzyme in extracts of tissues containing enzyme and inhibitor can give no reliable information as to the actual inhibition of the enzymes in the original intact tissue In the absence of a direct method for determining the percentage inhibition of the enzyme in tissues great caution must be used in evaluating the results of inhibition experiments

*Function in the gastric mucosa* Carbonic anhydrase is present in very high concentration in the parietal cells of the mammalian gastric mucosa (23, 24) All investigators agree that the parietal cells are concerned with the secretion of acid, but there is no agreement on the mechanism of acid secretion Hollander (54) and Bull and Gray (21) have ably collected and lucidly criticized most of the current theories The reviewer believes that although the mechanism of acid secretion remains a mystery the rôle of carbonic acid can be shown to be entirely independent of the mechanism postulated for the formation of acid.

Gray and his colleagues (50, 51) have shown that the juice collected from a dog's gastric pouch under histamine stimulation is made up of two components There is an alkaline, non-parietal secretion which contains 0 133 M chloride, 0 033 M bicarbonate, 0 155 M sodium, 0 007 M potassium and 0 004 M calcium ions The acid secretion contains 0 166 M chloride, 0 007 M potassium and 0 159 M hydrogen ions The acid secretion is in osmotic equilibrium with the plasma (44) The water in the gastric secretion appears passively as the result of the secretion of the other components of the juice, and no special mechanism need be postulated to account for its presence (27) The chloride ions are drawn from the blood as a secondary result of the secretion of hydrogen ions in order to satisfy the principle of the electrical neutrality of solutions, for they can be largely replaced by bromide ions (35) and to a smaller extent by iodide ions (29) while the rate of secretion of acid is unaffected. The presence of potassium ions has not been experimentally accounted for, but they are not a major constituent of the juice Remaining is the secretion of hydrogen ions to be explained, and the reviewer believes that no mechanism yet postulated is adequate to account for their secretion

No matter by what means the hydrogen ions are secreted the consequences of their secretion are the same ' In order to form a liter of acid secretion the gastric mucosa must withdraw one liter of water, 0 007 mol of potassium and

0 166 mol of chloride from the blood, and it must manufacture 0 159 mol of hydrogen ions. The difference between 0 166 mol of chloride and 0 007 mol of potassium is 0 159 mol chloride ions unaccompanied by positive ions which are withdrawn from the blood. In order to satisfy osmotic and ionic relations another negative ion must be secreted into the blood to replace the chloride ions. All the evidence derived from the study of the "alkaline tide" of the blood during acid secretion and from the study of arterial venous differences (20, 37, 68) points to the conclusion that the gastric mucosa replaces chloride with bicarbonate (28) If the bicarbonate is secreted by the mucosa it must be formed by the mucosa The bicarbonate doubtless comes from carbon dioxide produced and hydrated to carbonic acid within the mucosa In fact only negligible amounts of carbon dioxide formed in the mucosa during acid secretion can escape hydration within the mucosa, for as Gray has pointed out (49) if only one twentieth as much carbon dioxide as bicarbonate passes from the mucosa to the blood there would be no alkaline tide

Carbonic acid must be formed in amounts exactly equivalent to the amount of acid secreted, for the amount of chloride replaced is the amount which accompanies the hydrogen ions into the juice When the bicarbonate ions pass into the blood they leave behind in the mucosa a number of hydrogen ions exactly equivalent to those secreted in the juice These hydrogen ions are the source of hydrogen ions secreted The reviewer wishes it to be particularly clear that he is not suggesting that the hydrogen ions of the juice are those immediately derived from the ionization of carbonic acid (23) The true first ionization constant of carbonic acid is about $3 \times 10^{-4}$ at 38°C (82) The bicarbonate concentration in the cells cannot be much higher than that in the venous plasma Therefore if the acidity of the juice were to be reached by mass action the activity of carbonic acid in the cells would have to be about 14 M However, the mechanism secreting hydrogen ions must use hydrogen ions By secreting hydrogen ions into the juice the parietal cells would have an internal deficit of hydrogen ions This deficit is exactly filled by the hydrogen ions coming from the ionization of carbonic acid.

The concentration of chloride in dog blood is about 0 096 M per kgm water (61) If one liter of acid secretion is formed from $n$ liters of arterial blood the concentration of chloride in venous blood will be $(0\ 096\ n - 0\ 160)/(n - 1)$ mol per kgm water The A V difference will be $0\ 070/(n - 1)$ mol per kgm water Since 0 159 mol of bicarbonate passes into the blood for every liter of acid juice secreted, the bicarbonate A V difference will be $-0\ 159/n - 1$ The ratio $\Delta(HCO_3^-)/\Delta(Cl^-)$ will be $-2\ 27$ The seemingly paradoxical conclusion is reached that although ionic equivalence is satisfied the bicarbonate concentration of the venous blood rises more than the chloride concentration decreases. This conclusion has been experimentally verified (63) The changes in concentration will be distributed between the plasma and the erythrocytes on the basis of the well known equations defining the equilibrium conditions of blood. It can be shown that the decrease in plasma and cell chloride and the increase in plasma and cell bicarbonate will be proportional to the concentration

initially present The same is true of the water removed to form the secretion. As the result of the increase of bicarbonate concentration the blood pH will rise The rise in pH, removing hemoglobin still further from its isoelectric point, will cause the hemoglobin to be more negatively charged There will follow a secondary decrease in the cell chloride caused by migration of chloride ions to the plasma The result will be that the chloride secretion in the gastric juice will appear to come almost exclusively from the erythrocytes (68)

The foregoing argument shows that the formation of bicarbonate in the gastric mucosa is a necessary part of the secretion of acid It does not show that carbonic anhydrase is necessary In the present incomplete state of knowledge of the concentrations of the reactants within the parietal cell it is impossible to calculate exactly the rate of the uncatalyzed formation of carbonic acid, but rough calculations show that the uncatalyzed rate may provide enough carbonic acid Rats of average weight of 160 grams have an acid secreting mucosa weighing 500 mgm (33) The average carbonic anhydrase concentration in the mucosa is 2 2 E per mgm , or there is a total of 1100 E per whole mucosa. There is about 17 E per cmm of parietal cells (23) The whole mucosa therefore contains about 65 cmm of parietal cells Assuming no catalysis by buffer ions and no back reaction, the uncatalyzed rate of the hydration of carbon dioxide at 38°C is 0 13 ($CO_2$) mols per liter per second (82) The concentration of carbon dioxide in parietal cells is probably not much over 0 001 mols per liter The minimum uncatalyzed rate of hydration is $0\ 13 \times 10^{-3}$ mols per liter per second, or about $3 \times 10^{-5}$ mols per total volume of parietal cells per hour The average rate of gastric secretion is not over 0 2 ml per hour per 100 grams of rat or 0 32 ml per hour per total volume of parietal cells (92) The hydrogen ion concentration in this juice is not over 0 15 mol per liter Therefore, the rate of acid secretion is not over $5 \times 10^{-5}$ mols per hour This is of the same order of magnitude as the rate of the uncatalyzed reaction Considering the uncertainty of the assumptions, it can be tentatively concluded that if carbonic anhydrase is necessary only a small portion of the enzyme actually present is required. In animals such as the frog which secrete acid at a lower temperature there may be a greater difference between the rate of the uncatalyzed reaction and the rate of acid secretion, for the temperature coefficient of the rate of the uncatalyzed reaction is large (82)

The experimental test fails to demonstrate the necessity for carbonic anhydrase As stated above, the original claim that the inhibition of gastric acid secretion by thiocyanate is the result of the inhibition of carbonic anhydrase is wrong (25) Feldberg, Keilin and Mann (40) were unable to inhibit acid secretion in cats with sulfanilamide, and similar experiments using dogs failed to show an unequivocal inhibition (26) Thiophene-2-sulfonamide in a concentration of 25 mgm per cent, enough to inhibit most of the carbonic anhydrase in the gastric mucosa, does not inhibit acid secretion in cats (33) It therefore appears that enough, or almost enough, carbonic acid is supplied by the uncatalyzed fraction to allow the secretion of acid to be unimpaired by the inhibition of the enzyme.

It should be pointed out that the carbonic acid system does not occupy a central position in the acid secreting mechanism The function of the system is to satisfy the osmotic and ionic requirements imposed by the fact of the secretion of acid Even if more refined or decisive experiments do show that carbonic anhydrase is necessary for the functioning of the system the secretion of acid will not be explained.

Carbonic anhydrase is present in cells of surface epithelium and in some pyloric cells (23) These cells secrete an alkaline juice (43), and the enzyme may be a part of the secreting mechanism There has been no work on the subject.

*Function in the nervous system* Ashby (1, 2, 3, 4, 5, 6) has shown that carbonic anhydrase is present in the nervous system of various species in patterns which tend to be peculiar to the species There is usually a progressive rostral increase in the carbonic anhydrase content of the tissue, and the enzyme can be present in both white and grey matter Her original papers must be consulted for details of the distribution Considering the large variations in the distribution of the enzyme from one part of the nervous system to the other and from species to species, the reviewer believes that speculation on the function of the enzyme in the nervous system should not be entertained until the cellular locus of the enzyme is determined

The small amount of work on carbonic anhydrase in the nervous system has failed to demonstrate the necessity for the enzyme As Ashby points out the complete or almost complete absence of the enzyme from the spinal cord of hogs shows that carbonic anhydrase is not necessary for conduction of the nervous impulse or synaptic transmission at the lowest levels This conclusion is supported by the observations (32) that the excitability of cat peripheral nerve, the rate of conduction of the impulse and the recovery of excitability are utterly unaffected by the presence of sufficient thiophene-2-sulfonamide to inhibit carbonic anhydrase by more than 99 9 per cent Spinal reflexes in the cat and five different types of electrical activity of the rabbit's cortex are likewise unaffected by similar amounts of the drug The conclusiveness of these observations is impaired by the fact that it has been impossible to demonstrate that the enzyme is actually inhibited in the tissues by the percentage calculated from the inhibition constant

The claim (91) has been made that the action of sulfanilamide upon the resting potential of frog sciatic nerve under several experimental conditions is evidence that the reaction catalyzed by carbonic anhydrase has an important rôle in maintaining a polarized state of the axon The complete absence of carbonic anhydrase from frog sciatic nerve deprives the argument of much of its cogency

*Function in the pancreas* The pancreas forms two distinct external secretions One is the viscid secretion, elicited by pancreozymin and the vagal impulses which contains the digestive enzymes It has never been suggested that carbonic anhydrase is concerned with the formation of this juice The other secretion is the alkaline juice elicited by secretin It contains little or no enzyme activity, and it is isotonic with the blood (0 10) The concentrations of sodium

and potassium are similar to the concentrations of these ions in plasma, and experimental variations in the concentration of the ions in the plasma are reflected promptly and quantitatively in the pancreatic juice (56) These observations suggest that the pancreatic cells, unlike most other cells, are completely and freely permeable to these ions and that no special mechanism need be postulated to explain their secretion in the juice The concentrations of calcium, magnesium and phosphate ions in the juice are lower than the respective concentrations in the plasma, and these ions appear to filter through the pancreatic tissue with difficulty (10) The anions of the juice are almost exclusively chloride and bicarbonate ions (9) The proportion of the two anions varies with the rate of secretion of the juice, the bicarbonate concentration becoming higher and the chloride concentration lower as the rate of secretion increases At maximum rates of secretion the bicarbonate concentration in the juice is about five times the chloride concentration, the ratio of the anions being just the reverse of that in plasma The secretion of chloride and bicarbonate must be accounted for in any theory of mechanism of pancreatic secretion, and conceivably carbonic anhydrase could be a part of the mechanism

The question of whether carbonic anhydrase is a part of the mechanism depends upon the answer to the question concerning the origin of the bicarbonate in the juice On one hand it can be postulated that carbon dioxide derived from the metabolism of the pancreatic cells is hydrated to carbonic acid with the aid of carbonic anhydrase The carbonic acid upon ionization could furnish the bicarbonate of the juice On the other hand the bicarbonate secreted by the gland could be derived entirely from the plasma, the cells of the gland merely effecting the transfer of the ions from the plasma to the juice

The elegant and laborious experiments of Still and his colleagues (99) showed that there is invariably a decreased A-V carbon dioxide difference when the gland first begins to secrete under the stimulus of secretin In fact, the carbon dioxide in the venous blood may at first be lower than that in the arterial blood As secretion continues the A-V difference increases These observations suggest that at first part of the bicarbonate of the juice comes from the blood and part from metabolic activity of the gland Later, as the metabolism of the gland increases, the increased production of carbon dioxide by the gland is more than enough to supply that secreted in the juice Ball and his colleagues (11), however, believe that all the bicarbonate in the juice is derived from the plasma They injected bicarbonate containing radioactive carbon into the blood of dogs while collecting the pancreatic juice They found that the ratio of radioactivity in the pancreatic juice to that in the plasma was almost exactly equal to the ratio of the bicarbonate concentrations in the two fluids although the latter ratio was as high as five to one This result, they believe, proves that the bicarbonate of juice is derived mainly from the plasma and no more than 20 per cent of the bicarbonate in the juice is derived from the metabolic activity of the gland Although there is a remote possibility that the rate of exchange of the radioactive bicarbonate of the plasma with non-radioactive bicarbonate formed within the gland is sufficiently fast to account for the results, the con-

clusion must stand until it is disproved On the basis of these and other observations Ball and his colleagues have presented a theory of the mechanism of pancreatic secretion. This theory is that the pancreatic cells are differentially permeable to the ionic constituents of plasma When the pancreas is stimulated to secrete, sodium, potassium, and bicarbonate ions along with the water can freely pass through the cells to enter the pancreatic ducts The chloride ions cannot pass across the cell membranes as rapidly as the other ions and consequently, in order to maintain electro-neutrality, the bicarbonate ion replaces part of the chloride ion in the juice

This theory is admittedly incomplete It has several implications which are difficult to accept, one of which is that the only source of energy for the secretion can be the capillary blood pressure in the gland, and it fails to account for the large increase in metabolism occurring during secretion Nevertheless, it is the only current theory of the mechanism of pancreatic secretion, and it leaves no room for carbonic anhydrase. That carbonic anhydrase probably has no function in the gland is established by the complete failure of sulfanilamide in very high concentrations to inhibit the secretion of pancreatic juice or to affect the concentration of its constituents (100) This negative result, however, does not constitute proof of Ball's theory, for the uncatalyzed reaction may be fast enough to supply all the bicarbonate necessary if bicarbonate is formed within the pancreatic cells

The effect of thiophene-2-sulfonamide on the secretion of the pancreas has not been tested. Much work remains to be done on the mechanism of pancreatic secretion

*Function in the kidney* The brilliant and definitive work of Pitts and his colleagues (76, 77, 78) has entirely revised our concepts of the means by which the kidney forms acid urine Pitts has shown that the two older theories of the mechanism of the formation of acid urine, the bicarbonate reabsorption and the phosphate reabsorption theories (90) can account at best for only small fractions of the maximal titratable acidity of the urine The reabsorption of all the bicarbonate presented to the tubules can produce only 24 per cent of the maximum titratable acidity of the urine, and the reabsorption of all the alkaline phosphate can produce only 9 per cent of the titratable acidity In addition, Pitts has shown that the rate of reabsorption of phosphate is entirely independent of the acid base pattern of the blood and urine, and therefore the differential reabsorption of alkaline phosphate is not an *acidifying mechanism* The only means by which the tubules can form acid urine is to secrete hydrogen ions into the tubular fluid. There is no direct evidence concerning the mechanism of acid secretion by the tubules. Pitts (76, 78) has suggested that the tubules exchange hydrogen ions formed within the tubular cells for sodium ions from the tubular fluid There is an alternative possibility that the cells of one section of the tubules secrete hydrochloric acid while a lower section reabsorbs sodium and chloride ions The first suggestion has the advantage of simplicity and the economy of hypothesis, but no experimental test has yet been devised to distinguish between the two

Carbonic anhydrase is probably a part of whatever mechanism forms acid urine The enzyme is present in the kidney cortex and absent from the medulla (36) Since the distal tubules which probably form the acid (73) are in the cortex, the enzyme may be in these tubules, but this has not been established It is important that the cellular locus of the enzyme be determined The first experimental work on the function of the enzyme in the kidney was done by Hober (52) who showed that acid production by perfused frog kidneys can be completely and reversibly inhibited by adequate concentrations of a number of sulfonamide drugs having free sulfonamide groups capable of inhibiting carbonic anhydrase Irreversible inhibition of acid secretion by one of the drugs he employed has not been explained Related sulfonamide drugs not having sulfonamide groups and incapable of inhibiting carbonic anhydrase have no effect on the formation of acid urine by the frog kidney Similar results were obtained in acidotic rats in which it was shown that thiophene-2-sulfonamide injected in a dose sufficient to inhibit a large fraction of the carbonic anhydrase resulted in a transitory production of alkaline urine while the injection of the same amount of sulfanilamide but in a dose too low to inhibit carbonic anhydrase had no effect (33) Pitts (78) has shown that very high blood levels of sulfanilamide in dogs adequately studied by clearance methods reduce the titratable acidity of the urine However, Pitts has been unable to inhibit all of the acid production with sulfanilamide This might be accounted for by the facts, discussed in detail above, that sulfanilamide at even the highest doses does not completely inhibit carbonic anhydrase and that the uncatalyzed reaction at the temperature of the mammalian body may provide a significant fraction of the carbonic acid required by the kidney tubule

The early observations (71) that large doses of sulfanilamide result in the production of an alkaline urine and that they disturb the acid-base pattern of the body (12) are explained by the effect of the drug on the carbonic anhydrase of the kidney

One may speculate that the mechanism forming acid in the kidney is the same as that forming acid in the stomach If this be true, the rôle of the carbonic acid system in the kidney may be the same as that in the stomach, namely, to provide an endogenous source of hydrogen and bicarbonate ions which can satisfy the ionic and osmotic requirements imposed by the necessity of secreting acid The need for carbonic anhydrase in the kidney and not in the stomach may be explained by the facts that the kidney can secrete acid at a higher rate than can the stomach and that in the kidney as in the lungs the rate of flow of a fluid through a tube sets a time limit within which the required series of reactions must occur

*Functions in other tissues* The carbonic anhydrase present in the oviducts of hens (22) appears to have a function in the formation of egg shells The eggs of hens to which sulfanilamide has been fed have soft shells almost completely devoid of calcium carbonate (13) The formation of the hard shells of eggs may well depend upon the precipitation of calcium carbonate as the result of a high local concentration of bicarbonate ions resulting from the ionization

of carbonic acid which in turn depends upon the presence of sufficient carbonic anhydrase.

The fetuses of pregnant rats fed large doses of sulfanilamide or sulfapyridine show gross defects in calcification of the bones (14) There must be a cause other than the inhibition of carbonic anhydrase, for sulfapyridine is not an inhibitor of the enzyme Both drugs inhibit bone phosphatases (16), and such inhibition is the most likely cause of the defects

No experimental work has been done on the functions of carbonic anhydrase in fish and invertebrates The enzyme may be a part of the several secretory mechanisms which maintain osmotic equilibrium and the ionic pattern of blood in these animals, and some interesting results may be anticipated if work is done on this subject

## CONCLUSIONS

Carbonic anhydrase has been shown to play an important and essential rôle in erythrocytes, the kidney tubules and the shell forming organs of birds Despite serious attempts it has not been possible to demonstrate the necessity for the enzyme in the gastric mucosa, the pancreas and the nervous system It may have an important function in other tissues, particularly those of lower animals, but no experimental evidence has been obtained Much work remains to be done on the functions of the enzyme.

The reviewer believes that future workers on the functions of the enzyme might profitably be guided by the mistakes of previous workers These mistakes of which the reviewer has contributed his share, have seldom been mistakes of fact but rather mistakes of premature speculation based on incorrect assumptions.

The reviewer believes that the excesses of speculation and the bitterness of much polemical literature could be avoided if several rules were kept in mind. These rules are 1 an enzyme only speeds the rate at which a reaction attains equilibrium and has no effect on the equilibrium 2, whether an enzyme actually catalyzes a reaction depends on the presence and concentration of its substrate as well as on the presence of the enzyme, 3, the concentration of an enzyme in a particular cell or part of a cell is not evidence for the relative importance of the reaction the enzyme catalyzes, and 4, the amount that a catalyzed reaction is reduced by inhibition or destruction of an enzyme is not necessarily proportional to the degree of inhibition or destruction of the enzyme These rules have frequently been forgotten in research on carbonic anhydrase, if they can be remembered by future workers much of the apparently fruitless work on carbonic anhydrase will have served a purpose

## REFERENCES

References marked with an asterisk have been consulted only in abstract form

(1) Ashby, W J Biol Chem 151: 521 1943
(2) Ashby W J Biol Chem 152: 235 1944
(3) Ashby W J Biol Chem 155 671 1944
(4) Ashby W J Biol Chem 156: 323 1944
(5) Ashby W J Biol Chem 156: 331 1944

# PREPARATION AND CHEMISTRY OF ANTERIOR PITUITARY HORMONES

ABRAHAM WHITE

*Department of Physiological Chemistry, Yale University, New Haven, Conn*

The past ten years have witnessed the isolation of six hormones from anterior pituitary tissue Each of these has been prepared in a pure or a relatively pure form Although there may exist in the secretion of the anterior pituitary additional hormonal principles whose physiological effects are as yet undescribed, the specific chemical substances which have been isolated account for most of the recognized hormonal rôles assigned to the anterior pituitary The elucidation of these hormonal functions has come from observations of 1, the physiological effects of extirpation of the anterior pituitary gland, 2, the ameliorative effects resulting from injection of anterior pituitary tissue extracts into hypophysectomized animals, 3, the results produced by injections of excess of the gland's secretions, in the form of extracts, into the normal animal

The application of these methods of research has disclosed the diverse types of physiological activity which are under hormonal control of the anterior pituitary gland The isolation of six individual chemical substances has been a development of the physiological studies, and it has been demonstrated that each of the isolated products will restore in the hypophysectomized animal at least one of the physiological processes whose rate was greatly retarded as a consequence of hypophysectomy It is true that some of the effects produced by crude pituitary gland extracts, e g , the so-called diabetogenic and glycostatic actions, have not as yet been reproduced with any one of the highly purified products However, earlier studies with partially purified growth hormone suggested that this principle may be the regulator of processes responsible for the diabetogenic (122) and glycostatic (79) phenomena The restoration of a hypophysectomized animal to physiological normalcy by administration of suitable quantities of each of the six purified hormones has not yet been described Until this is done, it is not possible to state unequivocally whether the six isolated principles represent the total number of hormones normally produced by the anterior pituitary gland, or whether some as yet unrecognized anterior pituitary hormone remains to be defined in physiological and chemical terms

The anterior pituitary hormones which have been isolated in purified form are indicated below, and are given the name most commonly applied to each of these substances in the literature In parentheses are indicated some of the diverse names which have been used by various investigators to describe these anterior pituitary principles

1 The *lactogenic hormone* (prolactin, galactin, mammotropin) This hormone initiates the secretion of milk in the mammary gland which has previously hypertrophied as a result of increased circulating estrogen Prolactin also influences the functional activity of the corpora lutea, and may have a synergistic effect with estrogens on mammary gland development

2 The *adrenotrophic hormone* (adrenotropic hormone, adrenotrophin, adrenotropin, adrenocorticophin, adrenocorticotropin, corticotropin) This hormone exerts a trophic influence on the adrenal cortex, stimulating the secretion and growth of this endocrine gland

3 The *growth hormone* which controls the rate of skeletal growth and gain in body weight Acceleration of processes concerned with normal growth is seen when excessive amounts of this hormone are administered to the normal organism and results in enlarged skeletal structures and excessive weight increments

4 The *thyrotrophic hormone* (thyrotropic hormone, thyrotrophin, thyrotropin, which exerts a normal trophic influence on the thyroid gland Administration of the hormone causes disappearance of the thyroid colloid, the thyroid epithelium becomes hyperplastic, alveolar cavities collapse and mitotic figures appear in large numbers in the thyroid gland

5 The *follicle stimulating hormone* (FSH, gametogenic hormone, gametokinetic hormone, thylakentrin, prolan A, gonadotrophin I) This principle causes the growth of Graafian follicles preparatory to the release of ova, and stimulates the sperm-forming tissues of the testes

6 The *luteinizing hormone* (LH, interstitial cell-stimulating hormone, ICSH metakentrin, prolan B, gonadotrophin II) This hormone causes the formation of corpora lutea from preformed Graafin follicles, and stimulates the interstitial cells of the ovaries or testes

The discussion which follows will deal chiefly with the preparation, isolation and chemistry of the anterior pituitary hormones In order to characterize the degree of purification achieved by various fractionation procedures, and to evaluate the concentration of physiological activity effected it will be necessary to describe briefly the bioassay methods most commonly used in purification studies of each of the hormones In referring to certain of the pituitary principles, the endings trophic or trophin, rather than tropic and -tropin, respectively, have been used in this review The reasons for this choice have been carefully considered by Corner (39) and accepted by the Association for the Study of Internal Secretions

The earlier efforts to extract and concentrate the active pituitary principles have been adequately described by Van Dyke (162) It will be the purpose of this review to present for each of the pituitary hormones a brief description of the principal methods of bioassay, the more important contributions to the problem of purification and isolation, an outline of the methods by which isolation of a highly purified preparation has been achieved, and the data obtained from physical and chemical studies of the final products

The problems relating to the chemistry of anterior pituitary hormones are essentially problems of protein chemistry, since each of the purified hormonal principles appears to be a protein The methods of fractionation, isolation and characterization of these hormones are, therefore, the methods of protein researches Moreover, at this time it may be concluded that each of the active products is not an artifact or split product of a "macromolecule" of which each hormone might be a component portion This view is supported by the isola

# PREPARATION AND CHEMISTRY OF ANTERIOR PITUITARY HORMONES

ABRAHAM WHITE

*Department of Physiological Chemistry, Yale University, New Haven, Conn*

The past ten years have witnessed the isolation of six hormones from anterior pituitary tissue Each of these has been prepared in a pure or a relatively pure form Although there may exist in the secretion of the anterior pituitary additional hormonal principles whose physiological effects are as yet undescribed, the specific chemical substances which have been isolated account for most of the recognized hormonal rôles assigned to the anterior pituitary The elucidation of these hormonal functions has come from observations of 1, the physiological effects of extirpation of the anterior pituitary gland, 2, the ameliorative effects resulting from injection of anterior pituitary tissue extracts into hypophysectomized animals, 3, the results produced by injections of excess of the gland's secretions, in the form of extracts, into the normal animal

The application of these methods of research has disclosed the diverse types of physiological activity which are under hormonal control of the anterior pituitary gland The isolation of six individual chemical substances has been a development of the physiological studies, and it has been demonstrated that each of the isolated products will restore in the hypophysectomized animal at least one of the physiological processes whose rate was greatly retarded as a consequence of hypophysectomy It is true that some of the effects produced by crude pituitary gland extracts, e g , the so-called diabetogenic and glycostatic actions, have not as yet been reproduced with any one of the highly purified products However, earlier studies with partially purified growth hormone suggested that this principle may be the regulator of processes responsible for the diabetogenic (122) and glycostatic (79) phenomena The restoration of a hypophysectomized animal to physiological normalcy by administration of suitable quantities of each of the six purified hormones has not yet been described Until this is done, it is not possible to state unequivocally whether the six isolated principles represent the total number of hormones normally produced by the anterior pituitary gland, or whether some as yet unrecognized anterior pituitary hormone remains to be defined in physiological and chemical terms

The anterior pituitary hormones which have been isolated in purified form are indicated below, and are given the name most commonly applied to each of these substances in the literature In parentheses are indicated some of the diverse names which have been used by various investigators to describe these anterior pituitary principles

1 The *lactogenic hormone* (prolactin, galactin, mammotropin) This hormone initiates the secretion of milk in the mammary gland which has previously hypertrophied as a result of increased circulating estrogen Prolactin also influences the functional activity of the corpora lutea, and may have a synergistic effect with estrogens on mammary gland development

2 The *adrenotrophic hormone* (adrenotropic hormone, adrenotrophin, adrenotropin, adrenocorticophin, adrenocorticotropin, corticotropin) This hormone exerts a trophic influence on the adrenal cortex, stimulating the secretion and growth of this endocrine gland

3 The *growth hormone* which controls the rate of skeletal growth and gain in body weight Acceleration of processes concerned with normal growth is seen when excessive amounts of this hormone are administered to the normal organism and results in enlarged skeletal structures and excessive weight increments

4 The *thyrotrophic hormone* (thyrotropic hormone, thyrotrophin, thyrotropin, which exerts a normal trophic influence on the thyroid gland Administration of the hormone causes disappearance of the thyroid colloid, the thyroid epithelium becomes hyperplastic, alveolar cavities collapse and mitotic figures appear in large numbers in the thyroid gland

5 The *follicle-stimulating hormone* (FSH, gametogenic hormone, gametokinetic hormone, thylakentrin, prolan A, gonadotrophin I) This principle causes the growth of Graafian follicles preparatory to the release of ova, and stimulates the sperm forming tissues of the testes

6 The *luteinizing hormone* (LH, interstitial cell-stimulating hormone, ICSH, metakentrin, prolan B, gonadotrophin II) This hormone causes the formation of corpora lutea from preformed Graafin follicles, and stimulates the interstitial cells of the ovaries or testes

The discussion which follows will deal chiefly with the preparation, isolation, and chemistry of the anterior pituitary hormones In order to characterize the degree of purification achieved by various fractionation procedures, and to evaluate the concentration of physiological activity effected it will be necessary to describe briefly the bioassay methods most commonly used in purification studies of each of the hormones In referring to certain of the pituitary principles, the endings trophic or trophin, rather than -tropic and -tropin, respectively, have been used in this review The reasons for this choice have been carefully considered by Corner (39) and accepted by the Association for the Study of Internal Secretions

The earlier efforts to extract and concentrate the active pituitary principles have been adequately described by Van Dyke (162) It will be the purpose of this review to present for each of the pituitary hormones a brief description of the principal methods of bioassay, the more important contributions to the problem of purification and isolation, an outline of the methods by which isolation of a highly purified preparation has been achieved, and the data obtained from physical and chemical studies of the final products

The problems relating to the chemistry of anterior pituitary hormones are essentially problems of protein chemistry, since each of the purified hormonal principles appears to be a protein The methods of fractionation, isolation and characterization of these hormones are, therefore, the methods of protein researches Moreover, at this time it may be concluded that each of the active products is not an artifact or split product of a "macro[illegible]ule" of which each hormone might be a component portion [illegible]orted by the isola-

tion of identical protein hormones by use of different methods of fractionation in various laboratories, and by the physiological evidence of variations in the rate and the control of secretion of the individual hormones of the anterior pituitary gland

*The Lactogenic Hormone* The capacity of extracts of the anterior pituitary gland to initiate and maintain lactation was first demonstrated by Stricker and Grueter (158) in 1928 These observations have been confirmed and extended by numerous investigators, and the data have also been reproduced with highly purified preparations of lactogenic hormone (66, 134, 137, 160)

*Methods of assay* The methods of assay generally employed for the lactogenic hormone may be classified into two groups 1, lactation or mammary gland methods, based upon the ability of prolactin to initiate lactation in the estrogen-stimulated mammary gland of the hypophysectomized animal, and 2, crop-sac or crop-gland methods The latter are based upon the observation made in Riddle's laboratory (137) that when extracts containing the lactogenic hormone are administered intramuscularly to young pigeons, there occurs a proliferative hypertrophy of the crop-sacs This hypertrophy may be limited experimentally to only a small area of crop-sac in the pigeon by injecting small doses intradermally directly into the skin overlying the gland (121) The portion of the crop-sac stimulated by the intracutaneous technique is that directly under the site of injection This method has a high degree of sensitivity, approximately one-thousand times that of the intramuscular assay (117, 118), and is therefore useful for detecting small quantities of prolactin However, when adequate quantities of the lactogenic hormone are available for assay, the intramuscular injection method has been used since it appears to give more reliable and reproducible results (11, 66, 67, 118, 119, 137, 165)

*Extraction and purification of prolactin* The relative stability of the lactogenic hormone to chemical reagents has made it possible to extract this principle from anterior pituitary gland tissue by a variety of procedures These methods have been applied either to minced, fresh pituitary tissue or to acetone-desiccated pituitary glands Three types of extraction media have been employed· 1, aqueous, or aqueous-alcohol, alkaline extraction, 2, aqueous, or aqueous-acetone, acid extraction, and 3, chloroform extraction Beef and sheep pituitary glands have been most extensively used for the preparation of prolactin There are few data on prolactin from the pituitaries of other species

Bergman and Turner (17) made a careful study of four methods frequently employed for the extraction of the lactogenic hormone These methods differed in their initial extraction procedure One employed aqueous, alkaline extraction at low temperatures (74), a second was an acid, aqueous acetone procedure (120); the third involved extraction with glacial acetic acid (131), and the fourth used alkaline, aqueous alcohol (13) It was found that an alkaline 60 to 70 per cent ethanol extraction procedure was superior as judged by both total yield of prolactin and physiological activity per milligram of extracted solids

Extraction of pituitary tissue with alkaline solvents, however, removes not only more of the lactogenic hormone but also of other pituitary proteins, in-

cluding other hormones and non-hormonal proteins Therefore, while an alkaline extract is exceedingly useful as a means of obtaining a rather complete mixture of the pituitary hormones, the presence of all the active principles makes more difficult the separation in pure form of a particular protein hormone This difficulty is circumvented in either the acid or the chloroform extraction methods

Extraction of prolactin by acid solvents was suggested in 1933 by Lyons and Catchpole (120), who used acid acetone Two years later, glacial acetic acid was employed by McShan and Turner (131) The acid acetone technique has been used by many investigators, since it yields an extract containing significant quantities of prolactin which is relatively free of other pituitary proteins One major contaminant is the adrenotrophic hormone from which separation is readily effected Thus the same extraction procedure is useful as an initial step in the isolation of adrenotrophin Minced fresh pituitary tissue (either anterior lobes or whole glands) is extracted with approximately 60 to 80 per cent acetone which has been acidified with concentrated hydrochloric acid The final pH of the tissue-solvent mixture is between 1.0 and 2.0 Removal of the tissue and raising the acetone concentration of the extract to approximately 90 to 92 per cent results in the precipitation of the crude prolactin. The procedure was originally described briefly by Lyons and Catchopie (120) and by Lyons (117) and has since been presented in detail (111, 118, 165, 167)

Purification of the crude prolactin may be achieved in several ways Extraction with water separates the water soluble hydrochloride of the hormone from an insoluble residue (165, 167) Addition of acetone to 92 per cent concentration precipitates the prolactin At this stage the product is a mixture of several proteins One is prolactin, a second protein is the adrenotrophic hormone, another is a protein insoluble at pH 6 6, and a fourth component is a protein which is very soluble over the entire pH range Solution of the crude prolactin at pH 8.0, and adjustment to pH 6 6 in the presence of 0.05 saturation with ammonium sulfate removes the pH 6 6 insoluble protein From the supernatant, purified prolactin may be obtained by adjustment to pH 5 4 Further purification is obtained by repetition of this fractionation procedure This method of purification (165, 167) yields a product of maximum physiological activity and with physical and chemical properties similar to those found for crystalline prolactin preparations (165, 167) and for purified, amorphous products (111, 118)

Satisfactory purification of crude prolactin has also been achieved (111) by taking advantage of the differences in solubility between the lactogenic and adrenotrophic hormones at pH 3 0 in the presence of 0.36 molar sodium chloride Under these conditions, the lactogenic hormone is precipitated, whereas adrenotrophin remains in solution Further purification of prolactin is achieved by precipitation at its isoelectric point (pH 5 7) and repetition of the pH 3.0—sodium chloride procedure

Lactogenic preparations with maximum potency have also been obtained by purification of extracts initially made by extraction of fresh, ground pituitaries with chloroform (144) This procedure was developed in an attempt to circumvent the troublesome overlapping of hormone fractions encountered in other

methods, thus making more difficult the preparation of the hormones in pure form The chloroform technique offered the possibility of fractionating the starting material in such a way that a preparation of several hormones from one batch of glands might be readily achieved Sevag (145) had successfully used chloroform precipitation of proteins from aqueous solution in the purification of polysaccharides When ground, whole pituitaries were shaken with chloroform at a pH of 5 to 6 and the mixture subsequently centrifuged, three distinct layers were formed The lowest consisted of chloroform, in which most of the lipid substances of the tissue were present The layer above the chloroform was a gel which contained the bulk of the tissue proteins, together with prolactin and adrenotrophic hormone In the top clear aqueous solution were found thyrotrophin, gonadotrophins, pituitrin, and other easily soluble substances The lactogenic hormone was obtained from the chloroform gel by extraction with acid methanol and fractionation with sodium chloride The yield of purified prolactin was approximately ten times that obtained by previously described techniques

From highly purified prolactin preparations, a crystalline protein has been obtained (165, 167, 168) with properties indistinguishable from those of the amorphous preparations of the hormone The procedures described for the crystallization of the protein are apparently not satisfactory since they have not been uniformly reproducible Although crystallization of prolactin has been accomplished in one laboratory (143), lack of success has been reported in another (12) Further careful study should lead to a more satisfactory method of preparing not only crystalline prolactin, but certain other of the anterior pituitary hormones in crystalline form While crystallinity is not in itself an adequate criterion of purity of a protein, crystallization techniques are often useful as an aid in achieving further purification of proteins

*Properties of prolactin* Preparations of prolactin from both beef and sheep glands have been described which satisfactorily meet the criteria at present available for estimating the degree of purity of proteins (148) The most exacting of these criteria are electrophoretic and ultracentrifugal homogeneity, and satisfactory accord with the Gibbs phase rule in solubility behavior

The electrophoretic behavior of purified prolactin preparations from both sheep and beef pituitary glands has been studied by Li, Lyons and Evans (99, 100) and by Li, Simpson and Evans (111) The electrophoretic properties of crystalline prolactin from beef pituitary glands have been examined in the Tiselius apparatus by Shipley, Stern and White (150) and by White, Bonsnes and Long (167) The crystalline preparation has also been studied by the cataphoretic technique (167) based upon measurement of the electrophoretic mobility of microscopically visible quartz particles coated with an absorbed layer of protein The data obtained show that the lactogenic products from beef and sheep pituitary glands are indistinguishable in their electrophoretic behavior and have an isoelectric point at pH 5 7 However, a difference has been observed (100, 102) in the solubility behavior of prolactin prepared from beef glands as compared to the product obtained from sheep tissues, since, at

the same pH and salt concentration, the beef lactogenic hormone was less soluble than the preparation from sheep glands Indeed, it was reported that if solid sheep hormone was added to a saturated solution of the beef hormone, the former went into solution The greater insolubility of beef prolactin was attributed in part to its higher tyrosine content since the beef lactogenic product contained 5 7 per cent tyrosine whereas the sheep product has 4.5 per cent of this amino acid (104) Fleischer (65) has studied the solubility of prolactin in organic solvents and shown that at a pH below its isoelectric point the hormone is highly soluble in 99.8 per cent methanol and in 95 per cent ethanol

At the present time there is some difference of opinion regarding the molecular weight of the lactogenic hormone This difference apparently does not depend on the species from which the hormone was prepared. White, Bonsnes and Long (167) found a sedimentation constant of 2.8 S for prolactin Assuming a spherical shape for the molecule, this sedimentation constant indicated an approximate molecular weight of 35,000 for the protein hormone The same sample of prolactin was examined independently inthe laboratory of Prof J W Williams, at the University of Wisconsin The values reported (167) were 2 65 S for the sedimentation constant, $7.5 \times 10^{-7}$ for the diffusion constant, and 32,000 for the molecular weight On the other hand, osmotic pressure measurments (104) have indicated a molecular weight of approximately 26,000, and diffusion (sintered glass membrane) and viscosity measurements assigned a molecular weight of 22,000 to prolactin (91) From the two sets of data, the calculated dissymmetry constants ($f/f_0$) are 1.37 and 1.29 (91) The optical rotation of prolactin has also been established (91) The more important physical-chemical properties of prolactin are compiled in table 1

The differences in the molecular weight which have been reported for prolactin are difficult to reconcile On the basis of the sulfur and tryptophane contents (see below) of the lactogenic hormone, the calculated minimal molecular weights are in better agreement with the higher than the lower molecular weight values which have been reported for prolactin

The ultraviolet absorption spectrum of prolactin (170) is typical of that found (40, 89) for a number of proteins that are free of non amino acid residues which absorb in the region of 2500 A° to 3000 A° The broad band with a maximum at 2800 A° is evidence for the presence of aromatic amino acids in prolactin.

The elementary composition of prolactin is typically that of a protein It contains 16.5 per cent nitrogen (165) and has a relatively high sulfur content, 1.8 (104) to 2.0 (167) per cent On the basis of its sulfur content, a minimal molecular weight of approximately 16,000 may be calculated. Qualitative tests for phosphorus, carbohydrate, and sulfhydryl groups are negative The amino acid composition has not as yet been extensively studied The cystine content has been reported as 3.0 (68, 104) 3 1 (92) and 3 4 (167) per cent, and a value of 4.3 per cent was found (92) for methionine Within the limits of experimental error, the cystine and methionine contents of both beef and sheep lactogenic hormones are the same and account for the total sulfur in the hormone Other amino acid values reported are arginine, 8.3 per cent (104), tryptophane, 1.3 per cent

(101, 167), and 2 5 per cent (104), and tyrosine, 5 5 per cent (167) and 5 7 per cent (101, 104) for the beef lactogenic hormone The values reported for the hormone from sheep pituitaries were similar with the exception of that for tyrosine, which was found to be 4 5 per cent (101, 104) The difference in tyrosine

TABLE 1

*Some physical-chemical properties of prolactin**

*Solubility*
In 0.357 M NaCl at pH 2.25
0 606 g /l (sheep) (102)
0 316 g /l (beef) (102)

*Isoelectric point*
pH 5 60 (150)
pH 5 65 (165, 167)
pH 5 70 (99)
pH 5 73 (100)

*Molecular weight*
From osmotic pressure, 26,500 (104)
From diffusion and viscosity, 22,000 (91)
From sedimentation-diffusion, 32,000 (167)

*Diffusion constant* ($D_{20w}$)
Sintered glass membrane, $9\ 0 \times 10^{-7}$ (91)
Free diffusion, $7.5 \times 10^{-7}$ (167)

*Partial specific volume* ($V_1$), 0 721 (91)

*Viscosity coefficient* $\left(\left(\frac{\eta}{\eta_0} - 1\right)1000/cV_1\right)$, 6 65 (91)

*Dissymmetry constant* ($f/f_0$)
Viscosity data, 1.29 (91)
Sedimentation-diffusion, 1 37 (91)

*Optical rotation*
$[\alpha]_{25°}^{Na} = -40\ 5°$ (91)

* The numbers in parentheses are bibliography references Except for solubility differences between beef and sheep prolactins, the other physical-chemical properties appear to be independent of species source of the hormone

content of the hormones from the two species, and the difference in solubility, mentioned previously, are the only dissimilarities which have as yet been established in comparisons of the beef and sheep lactogenic hormones Despite these detectable physical and chemical differences, the hormones from beef and sheep pituitaries are immunologically indistinguishable (19)

There is good agreement among the data for the amino acid composition of the

lactogenic hormones prepared in different laboratories, with the exception of the tryptophane values A tryptophane content of 1.3 per cent was obtained when analysis was conducted on completely hydrolyzed prolactin (101, 167), a higher value of 2.5 per cent was found by a glyoxylic acid method (147) applied to the intact protein. Brand (24) has confirmed the lower tryptophane value by finding 1 2 per cent of this amino acid in a preparation of prolactin supplied by the writer The procedure employed in Brand's laboratory was a photometric technique (25) based on the method described by Lugg (116) The data obtained from amino acid analysis of prolactin are assembled in table 2

*Effect of physical and chemical agents on prolactin* a *Heat and pH* Earlier studies (137) of the heat stability of prolactin suggested that the hormone was relatively thermostable when heated in a boiling water bath for one hour at pH 7.0 to 8.0, but less stable at other pH values However, the reported relative heat stability of the lactogenic hormone, as in the case with other biocatalytically active proteins, may be influenced by a variety of factors The presence of other

TABLE 2

*Some amino acid analyses of prolactin**

| | |
|---|---|
| Tyrosine | |
| Beef prolactin | 5 5% (167), 5 7% (101, 104) |
| Sheep prolactin | 4 5% (101 104) |
| Tryptophane | 1 2% (24) 1 3% (101 167), 2 5% (104) |
| Cystine | 3 0% (68 104) 3 1% (92), 3 4% (167) |
| Methionine | 4 3% (92) |

* The numbers in parentheses are bibliography references Except for indicated differences in tyrosine contents beef and sheep prolactins appear to have the same percentages of the other amino acids which have been studied

proteins may result in an increased resistance of each of the proteins in the mixture toward labilizing agents Moreover, the concentration of protein and inorganic electrolyte and the pH of the solutions examined may influence the nature of the data obtained Experiments with purified prolactin (167) indicated that this hormone may be classed as a heat-labile substance Destruction of hormonal activity by heat proceeded most rapidly in solutions more alkaline than pH 11 0, and exposure of a 0.04 per cent solution of prolactin to 100 degrees centigrade for 30 minutes resulted in considerable loss of biological activity over the entire pH range In alkaline solution, this heat-lability is accompanied by a splitting of labile sulfur from the hormone, a significant degree of hydrolysis of the protein occurs at elevated temperatures in either acid or alkaline medium

b *Hydrolysis* The hydrolysis of prolactin with boiling 20 per cent hydrochloric acid gave (167) an hydrolysate which retained no evidence of lactogenic activity even when assayed at levels approximately 400 times that [illegible] o produce a physiological response with the unhydrolyzed [illegible] with pepsin or trypsin also resulted in rapid inactivation of [illegible] 5, [illegible] of [illegible]

occurred before there had been hydrolysis of the protein into fragments which were no longer precipitable by trichloroacetic acid

c *Denaturing agents* Li, Lyons and Evans (103, 104) and Li (91, 93) have studied the effects of urea and of a commercial detergent on the properties of the lactogenic hormone Using viscosity measurements as an index of alterations in the physical state of the protein, it was demonstrated that, in the presence of 3 0 molar urea, the relative viscosity of the lactogenic hormone is greatly increased However, osmotic pressure studies revealed that the hormone does not dissociate in 6 66 molar urea solution Unfortunately, it was not possible to determine the biological activity of the hormone in urea solutions, but after removal of the urea by dialysis, the physiological activity was found to be the same as that noted before urea treatment In the case of urea, therefore, it was not possible to establish unequivocally whether the denaturating agent also affected physiological activity Similar increases in viscosity of solutions of the protein were also produced with solutions of detergents, a distinct lowering of the biological activity of the hormone resulted The reversibility of these alterations could not be assessed, since efforts to remove the detergent from the protein were unsuccessful Inasmuch as the biological activity of prolactin appears to depend in part on the presence of intact phenolic hydroxyl and unsubstituted amino groups in the molecule (see page 583), the loss of lactogenic activity in the detergent studies might have been due to interactions between the detergent and either or both of these groups

d *Action of ketene* Ketene has been widely employed as a reagent for examining the effect of substitution of a protein's free amino groups and the phenolic hydroxyl groups of tyrosine on the activity of proteins which exhibit one or another type of biocatalytic or physiological behavior Acetylation of proteins with ketene has the advantage that the reaction may be conducted under conditions (aqueous solutions and low temperatures) which do not alter appreciably the native state of the protein It has been generally assumed that ketene effects a degree of selective acetylation in the case of most proteins, since amino groups have been reported to react much more rapidly than do hydroxyl groups (18, 80, 81, 157) However, it should be emphasized that investigations of the reaction of proteins with ketene have concentrated attention on measurements of the degree to which free amino or phenolic hydroxyl groups have been substituted, and have ignored completely the possible acetylation of aliphatic hydroxyl groups present in certain amino acids, i e , serine and threonine Inasmuch as it is unknown at the present time whether these two amino acids have a rôle in determining the biocatalytic activity of certain proteins, the results obtained by the use of ketene as an acetylating agent and by the measurement of the degree of substitution of amino and phenolic hydroxyl groups are not possible of interpretation solely in terms of the contribution of these groups to the activity of the protein Although data and conclusions obtained from studies of the inactivation of protein hormones by ketene will be presented in this review, in no single case has the action of ketene been shown to be limited only to those protein groupings whose biological importance is being assessed

In a preliminary report Li, Simpson and Evans (106) observed that the treatment of prolactin with ketene at 20 degrees centigrade for 5 minutes resulted in acetylation of all the free amino groups of the hormone and a complete loss of physiological activity It was not demonstrated that substitution of groups other than free amino groups had not occurred These additional data seemed necessary, since proteins may differ in the reaction rates which different groups in the molecule may exhibit with ketene Indeed, in a recent complete publication, Li and Kalman (98) have observed that the reaction of prolactin with ketene proceeded in an unexpected manner The acetylation of up to 20 per cent of the phenolic hydroxyl groups of tyrosine residues in prolactin was reported to have occurred without acetylation of any free amino groups in the protein This type of protein substitution could be achieved by treatment with ketene for three minutes at pH 7 0 in phosphate buffer, the product obtained exhibited the full biological activity of the original prolactin Under these conditions, therefore, ketene appeared to react with the phenolic groups of tyrosine residues in the lactogenic hormone molecule more rapidly than with the amino groups On the other hand, in acetate buffer at pH 4 0, the extent of acetylation of both amino and phenolic groups after five minutes of treatment was practically identical, 35 per cent of each type of grouping being acetylated. This product had only one fifteenth the biological activity of the unacetylated hormone In other preparations in which 70 per cent of the amino groups and 70 per cent of the phenolic hydroxyl groups were acetylated, the biological activity was reduced to almost the vanishing point The authors concluded that the decrease in crop stimulating potency of the acetylated products was due to a substitution of the free amino groups of the protein hormone This conclusion that the amino groups are essential for the biological activity of the hormone was given further support by the demonstration that hydrolysis of the phenolic oxygen-acetyl groups in the acetylated, inactive protein under conditions known not to inactivate the lactogenic hormone (pH 11 for 10 min ) did not restore the biological activity This would be the expected result if the decreased potency of acetylated prolactin preparations was unrelated to the degree of involvement of the tyrosine residues of the molecule, but dependent solely on the free amino groups

c *Action of other agents primarily affecting amino groups* Prolactin has been treated with a number of reagents which chiefly affect the free amino groups of the protein However, in the case of almost each of these reagents, adequate data have not been presented to establish that only free amino groups have been altered by the reagent employed The data, therefore, must again be interpreted with a considerable degree of reservation Lactogenic activity was destroyed by treatment with sodium nitrite, formaldehyde, benzoyl chloride or by diazotization (125) Groups other than free amino groups were undoubtedly involved in these reactions Treatment with nitrous acid at low temperatures also resulted in complete inactivation of the hormone (105) Even at low temperatures, however, alterations other than deamination of the free amino groups occur when proteins react with nitrous acid (171)

Treatment of prolactin with phenylisocyanate at low temperatures yielded a phenylureido derivative which had only about 10 per cent of the biological activity of the original hormone (23) The specificity of phenylisocyanate as a reagent for only the free amino groups of proteins has been seriously questioned (132)

f *Iodination* The effect of iodination on the biocatalytic activity of various proteins has been employed as an approach to determining the contribution which the tyrosine residues of the protein make to the specific activity However, these studies have not given adequate consideration to other types of reactions which may also occur when proteins are treated with iodine Changes in the protein of an oxidative nature may ensue, as may the iodination of protein groupings (15) other than the aromatic nuclei of the tyrosine residues The reaction of prolactin with iodine has been studied in some detail by Li, Lyons and Evans (103) Iodination in phosphate buffer of pH 7 0 at 20 degrees centigrade for one hour resulted in an introduction of iodine into the phenolic portions of the tyrosine residues of prolactin, with an accompanying complete loss of hormonal activity

g *Effect of thiol compounds* The reducing action of thiol compounds on prolactin produced a varying degree of inactivation, depending on the extent of reduction (72) Treatment of a solution of prolactin with a 40-fold concentration of cysteine transformed the hormone into an insoluble protein which, when redissolved under conditions which prevented autoxidation, was as biologically active as the untreated hormone However, when a 200-fold quantity of cysteine was employed to reduce prolactin, inactivation occurred Thioglycolic acid was approximately 50 times more effective than cysteine in causing inactivation of the lactogenic hormone

In summarizing the data on prolactin, it is evident that the purity of the hormone has been established by satisfying the criteria usually employed in studies of protein purity There is no evidence for the presence in the molecule of residues other than amino acids The physical-chemical properties of prolactin have been rather well characterized, and a few analytical data for amino acid composition are available The hormone is a heat labile protein, rapidly inactivated by mild hydrolysis or by reagents which have been assumed to affect chiefly the free amino, or disulfide groups of the protein Although acetylation of the phenolic hydroxyl groups of the tyrosine residues has been reported not to diminish the biological activity of prolactin, iodination of these residues was accompanied by a rapid destruction of lactogenic potency

*The Adrenotrophic Hormone* The basic experimental evidence that has led to the acceptance of a distinct hormonal secretion of the pituitary gland with a trophic influence on the adrenal cortex was provided by the investigations of P E Smith (153, 156) This investigator described the atrophy of the adrenal cortex in the hypophysectomized rat and the adrenal cortical repair produced by implantation of fresh, living hypophyseal tissue As a consequence of these studies, the question arose as to whether adrenal cortical atrophy in the hypophysectomized animal was a primary effect due to the lack of a specific trophic

hormone elaborated by the pituitary for the purpose of maintaining the adrenal cortex, or a secondary result due to a metabolic disturbance (lowered thyroid functioning) accompanying hypophysectomy However, a few years prior to Smith's classical work, Evans and his colleagues (44) were able to extract from pituitary tissue potent growth promoting preparations, some of which caused adrenal hypertrophy This important contribution established that adrenal cortical stimulation was not dependent upon the presence of whole pituitary tissue but could be elicited by fractions of this tissue The investigations in Evans' laboratory had progressed enough by 1933 for him to state (45) " it would appear inevitable for us to recognize the existence of an adrenalotropic hormone, the properties of which as distinguished from thyrotropic, lactogenic, and other possible hormones will emerge, I trust, from the research of the three or four laboratories engaged in this study in the course of the next few months " In the same year, Collip, Anderson and Thomson (37) obtained from pituitary tissue extracts a fraction which in relatively small doses would produce repair of the adrenal cortex of the hypophysectomized rat Since the material was free of thyrotrophic hormone, the data confirmed the existence in pituitary extracts of a distinct adrenotrophic principle

*Methods of assay* Several methods of assay of the adrenotrophic hormone have been proposed Two of the most reliable of these (96, 141, 151) employ the hypophysectomized animal in which variable internal sources of the hormone have been removed One assay is based upon the ability of the product being tested to repair the atrophied adrenals found in rats several weeks following pituitary removal The other evaluates the potency of a preparation in terms of its capacity to prevent a decrease in the size of the adrenal cortex of the rat following hypophysectomy The first procedure is thus termed the repair, and the second the maintenance, method of assay

*Isolation of adrenotrophic hormone* Almost ten years elapsed between the publication of physiological evidence for the presence in pituitary tissue of a specific adrenotrophic principle and the laboratory production of preparations containing significant amounts of this hormone Collip and his associates (35, 37) used alcohol and acetone filtrates which were obtained after removal of precipitated thyrotrophic hormone These filtrates were concentrated at low temperatures and the solution was saturated with ammonium sulfate The precipitate which formed was extracted with one per cent ammonia, the extract dialyzed and the proteins precipitated with alcohol The precipitate was extracted with dilute alkali, and isoelectric fractionation yielded a product which was insoluble at pH 6 0 and was free of thyrotrophic hormone and rich in adrenotrophic factor

Two procedures which assume interesting properties for adrenotrophin have been proposed for the preparation of this hormone One method assigns to adrenotrophin a considerable degree of heat stability The other preparative approach suggests that adrenotrophic hormone activity may be associated with ultrafilterable moieties Collip (36) has suggested extraction of fresh, defatted and alcohol-dehydrated pituitary tissue with 0.25 per cent acetic acid for 15

minutes Hydrochloric acid was then added to 0 25 per cent concentration and the mixture boiled for 12 hours Neutralization of this extract to pH 4 0 yielded a solution which contained adrenotrophic hormone The use of elevated temperatures has also been proposed in a Dutch patent (135) Pituitary extracts were prepared by extraction with water solution at pH 6 0 to 6 6 for 45 minutes at boiling temperature The procedure was reported to decompose heat labile hormones such as the gonadotrophins After filtration of the extract, further purification was achieved by adsorption of the hormone on active carbon and elution with water The bioassay method employed was not indicated, and hence it is difficult to evaluate the product obtained Although it has been the writer's experience that methods employing heat treatment of pituitary tissue have not yielded potent adrenotrophic hormone products, it should be noted that the purified adrenotrophic hormone prepared from sheep pituitary glands (see p 587) has been reported to exhibit a significant degree of heat stability in dilute hydrochloric acid solution (96)

The first indication that the adrenotrophic factor could be ultrafiltered was presented by Anselmino, Hoffmann and Herold in 1934 (5) These investigators reported that adrenotrophin could be separated from gonadotrophic, thyrotrophic and lactogenic activities by ultrafiltration of the adrenotrophic hormone through 8 per cent acetic acid-collodion membranes It is unfortunate that the measure of adrenotrophic activity was based on changes in size and lipid concentration of the adrenals of castrated infantile mice Positive assay results under these circumstances could have resulted from non-specific stimulation of the animals' own pituitary (166) It would be of interest to assay similar products in the hypophysectomized rat The data of Tyslowitz (161) also indicated that a dialyzable fraction containing adrenotrophic hormone activity could be prepared from pituitary glands Acetone-dried hog pituitary powder was extracted with glacial acetic acid and the extract dialyzed for two weeks against an equal volume of water Assay of the dialysate in hypophysectomized rats showed the presence of adrenotrophic activity In other experiments, clear and almost colorless ultrafiltrates were obtained with the aid of cellophane membranes and pressure filtration Almost half of the solids of the initial extract were ultrafiltrable and represented 38 per cent of the total adrenotrophic activity of the extract The material remaining in the dialysis bag contained 28 per cent of the total activity The ultrafiltrates were free of gonadotrophic and thyrotrophic hormones By sodium chloride and ammonium sulfate fractionation at pH 4 5 of the ultrafiltrates, Tyslowitz obtained a preparation with marked adrenotrophic hormone potency The product gave the usual protein color reactions, was precipitated from 0 1 per cent solutions by phosphotungstic or phosphomolybdic acids, but not by trichloroacetic or picric acids Adrenotrophic hormone activity was not destroyed by boiling a solution of the preparation for 10 minutes

During the course of studies on prolactin, acid-acetone extracts which yielded crude prolactin preparations were also shown to have a significant concentration of adrenotrophin Lyons (117) precipitated an adrenotrophic fraction from a slightly alkaline solution of crude prolactin by adjusting the pH to 6 5 This

product exhibited adrenotrophic activity when assayed in the hypophysectomized rat and in the immature male rat (133) As a result of these observations it was erroneously accepted that the adrenotrophic hormone had an isoelectric point at pH 6.5 Bates, Riddle and Miller (14) observed the high degree of solubility of adrenotrophic hormone fractions in 60 per cent ethanol and in water at pH 3.5 On dialysis of aqueous solutions at pH 3.5, a precipitate formed which was slightly more potent in adrenotrophin than was the remaining solution

Preliminary announcements from two laboratories of the isolation of purified adrenotrophic hormone (110, 140) were followed by the two complete publications appearing at the same time (96, 141) Of considerable interest is the fact that two groups of investigators, working independently, have applied considerably different fractionation procedures to pituitary tissue of two different species and obtained what appears to be identical protein hormone products The procedure of Li, Simpson and Evans (96) began with an acid-acetone extraction of fresh sheep pituitary glands and prepared what may be described as crude prolactin Fractionation of this product was based on the differences in solubility of adrenotrophin and prolactin in the presence of sodium chloride Prolactin was precipitated from aqueous solution at pH 3.0 in the presence of 0 54 molar sodium chloride. After removal of this precipitate, adrenotrophin was precipitated from the supernatant by increasing the salt concentration to 1.35 molar Sayers, White and Long (141) also used as starting material crude prolactin which was, however, prepared from hog pituitaries This tissue was chosen because of the report (10) that hog pituitaries were higher in adrenotrophic hormone content than pituitary tissue from several other species examined It is interesting to note that the yield of crude prolactin from hog glands was approximately two and one-half times that from a corresponding weight of beef pituitaries (167) This was probably in part related to the higher adrenotrophin content of the hog tissue, although no careful studies have been made of the prolactin content of the hog pituitary The isolation of purified adrenotrophin from hog crude prolactin was achieved by careful adjustments of hydrogen ion concentration, advantage being taken of differences in isoelectric points of the two hormones (prolactin, 5 7, adrenotrophin, 4 7) The yield of purified adrenotrophic hormone from hog pituitaries was approximately five-fold that from sheep tissue

The purity of the isolated adrenotrophins from the two species has been established by ultracentrifugal, electrophoretic and solubility studies (96, 141) [1] Both groups of investigators obtained a molecular weight of 20,000 for their hormone preparations The product from sheep glands was reported (96) to exhibit a considerable degree of heat stability at 100 degrees centigrade in buffer at pH 7.5, or in 0.1 molar hydrochloric acid. However, biological activity was lost when adrenotrophin was heated in 0 1 molar sodium hydroxide solution, precipitated by trichloroacetic acid, or digested by trypsin Adrenotrophic activity of the protein hormone was little affected by the digestive action of pepsin Indeed, adrenotrophic hormone activity was claimed to be unimpaired

[1] Also unpublished data

when 37 per cent of the original protein was no longer present as a result of peptic action Li, Simpson and Evans (113) have also studied the reaction of purified adrenotrophin with ketene, nitrous acid, formaldehyde and iodine, and correlated the resulting chemical and biological alterations As in the case of prolactin (98), it was demonstrated that ketene reacted more slowly with amino groups than with phenolic hydroxyl groups in acetate buffer at pH 4 0 However, in phosphate buffer of pH 7 0, five minutes, treatment with ketene acetylated 50 per cent of the amino groups and one-fifth of the tyrosine phenolic groups It was difficult from the type of products obtained to assess accurately the relative contribution of the two types of groups to the biological activity Since adrenotrophin potency declined as acetylation was prolonged, it was concluded that both the phenolic hydroxyl and amino groups are essential for the biological action of the adrenotrophic hormone Reaction with nitrous acid for 30 minutes completely inactivated the hormone, as did treatment with formaldehyde These data further indicated the essentiality of the free amino groups The biological importance of the tyrosine residues was further suggested by the demonstration that the degree of inactivation of the hormone by iodination was correlated with the amount of iodine absorbed by the protein solution The interpretations of the data obtained in these inactivation studies of adrenotrophin are limited by considerations previously discussed

Recently, Li (94) has found that adrenotrophin isolated from sheep pituitaries contained 1 93 per cent methionine and 7 19 per cent cystine These values for the sulfur-containing amino acids satisfactorily accounted for the total sulfur, 2 32 per cent, of this hormone The more important analytical data for adrenotrophin are summarized in table 3 Other amino acid analyses and further inactivation studies would be important contributions to the knowledge of adrenotrophin, particularly in view of the suggestive evidence that a moiety with similar physiological activity may be obtained from this protein hormone by ultrafiltration or by peptic digestion

*The Thyrotrophic Hormone* In 1911, Ascoli and Legnani (8) demonstrated that hypophysectomy in the mammal was followed by atrophy and inactivity of the thyroid gland This was established independently by Aschner (7), and in 1914 Adler (2) was able to prevent metamorphosis and to cause thyroid atrophy by destroying the pituitary in tadpoles Two years later, Allen (3) and Smith (152) independently showed that in tadpoles the ablation, in the late egg stage, of the ectodermal bud destined to form the anterior, intermediate and tuberal lobes of the hypophysis, resulted in a failure of the thyroid to undergo normal development A study of the injection or transplantation of the various lobes of the pituitary revealed that use of the anterior, but not of the other lobes, induced reparative effects in the thyroid The conclusion was drawn that the lack of thyroid development following ablation of the ectodermal bud was due not to the absence of the entire pituitary but rather to the loss of its anterior lobe component It was further observed that the feeding of fresh anterior lobe tissue had no restorative effect on the thyroid, whereas parenteral administration of the tissue stimulated thyroid development

The first thyroid-stimulating pituitary extracts were merely crude acid, alkaline or saline extracts of pituitary gland tissue (114) Although these extracts were largely mixtures of all pituitary hormones, the investigations were of value in their demonstration that the guinea pig was an exceedingly sensitive test object for assay of the pituitary thyrotrophic hormone

*Methods of assay* Several satisfactory biological methods are available for assessing the thyrotrophic hormone potency of pituitary preparations The most reliable methods depend upon the direct effect of the hormone on the thyroid gland and evaluate activity in terms of alterations in histology of the thyroid gland, changes in iodine content of the thyroid, or increases in thyroid weight The chick and the guinea pig are sensitive test animals

TABLE 3

*Analytical data for adrenotrophin*

| | HOG ADRENOTROPHIN (141) | SHEEP ADRENOTROPHIN (94 %) |
|---|---|---|
| Yield in mgm from 1 kgm fresh tissue | 350 | 70 |
| Minimum effective dose μg† | 25 | 25 |
| $S_{21}$ | 2 07 S | 2 08 S |
| $D_{20}$ | not determined | $10\ 4 \times 10^{-7}$ |
| Estimated molecular weight | 20,000 | 20 000 |
| Isoelectric point pH | 4 7 | 4 7 |
| Carbon | 50 64% | 46 35% |
| Hydrogen | 6 23% | 5 89% |
| Nitrogen | 15 47% | 15 65% |
| Sulfur | 2 33% | 2 30% |
| Cystine | not determined | 7 19% |
| Methionine | not determined | 1 93% |
| Carbohydrate | absent | absent |
| Heat stability‡ | not determined | + |

* The numbers in parentheses are bibliography references

† For a discussion of the comparative biological activities of the two preparations see reference 166

‡ + indicates heat stability under specific conditions

*Preparation of thyrotrophic hormone* The literature on the preparation of thyrotrophin is considerably more extensive than that dealing either with prolactin or with adrenotrophin The thyrotrophic hormone has been found in the pituitaries of almost all animals studied The concentration of the hormone in the gland showed a species variation, usually in direct relation to the activity of the animal's thyroid gland (159) The most commonly employed starting material for the preparation of the thyrotrophic hormone has been beef pituitary tissue Aron (6) reported that one milligram of fresh beef pituitary gland tissue, injected into the guinea pig, produced definite histological alterations in that animal's thyroid gland Sheep pituitaries are as high, or higher in their thyrotrophin content (20) whereas dog and horse glands have less of this hormone (83) Considerable variations may be found in reports of the thyro-

trophin content of pituitary tissue since bioassays have frequently been conducted on acetone-dried pituitary glands Jorgensen and Wade (86) observed that acetone drying of pituitaries may result in a loss of approximately 50 per cent of the thyrotrophic potency originally present in the glands

It might be expected that the whale pituitary would be a good starting material for preparation of pituitary hormones The pituitary in the whale is large, and the anterior lobe is easily obtained free from other portions of the hypophysis However, the yearly prewar catch of 10,000 whales would yield but 42 kilograms of dried pituitary powder, and this is equivalent in thyrotrophic activity to only about 26 kilograms of dried beef pituitary powder (21) It is interesting to note that while dilute acetic acid efficiently extracts thyrotrophin from beef pituitary powder, this solvent extracted little of the hormone from whale pituitary powder (21) Dilute alkaline extraction, on the other hand, gave solutions highly potent in thyrotrophic activity There is as yet no explanation of these observations

Among the first purified preparations of thyrotrophic hormone was that made by Janssen and Loeser (83) in 1931 A physiological saline extract of acetone-dried tissue was treated with sulfosalicylic acid solution Removal of the precipitate which formed yielded a solution showing marked thyroid-stimulating activity when injected intraperitoneally in guinea pigs No activity was evident following oral administration or after heating of the solution A year later, the same laboratory (115) reported very efficient extraction of thyrotrophin from pituitary tissue with aqueous solutions of pyridine, diethylamine or ammonia Protein impurities were removed with 20 per cent trichloroacetic acid, and thyrotrophic activity then precipitated from the filtrate by the addition of excess acetone Further purification was obtained by extracting the dried acetone precipitate with methanol to remove traces of yellow-colored material The residue was extracted with water, addition of excess acetone to the extract yielded a white powder equivalent in activity (82) to about twenty times its weight of the dry pituitary powder used as starting material Rowlands and Parkes (139) also succeeded in obtaining potent thyrotrophic hormone powders by extraction of acetone-dried pituitary powder with 50 per cent aqueous pyridine Extraction of thyrotrophin from anterior pituitary tissue by ammoniacal solutions was also used by Anderson and Collip (4) Growth hormone and other impurities were removed by adsorption on calcium phosphate Saturation of the calcium phosphate filtrate with ammonium sulfate gave a precipitate which had thyrotrophic activity This precipitate was dissolved in water and further purification of thyrotrophin achieved by alcohol or acetone precipitation

Acetic acid has been employed to extract thyrotrophin from acetone-dried pituitary tissue (87), addition of picric acid to these extracts precipitated the active material The picric acid was removed by the use of acid alcohol or by solution of the picrate in aqueous ammonia and precipitation of the protein with acetone Repetition of the picric acid step finally gave a highly potent product in a yield of 12 to 18 grams per kilogram of acetone-dried anterior lobe powder Dilute acetic acid was also employed as the initial solvent by Lambie and

Trikojus (88), who removed protein impurities from the extract by treatment with sulfosalicylic acid Thyrotrophic activity was then precipitated with sodium tungstate The tungstate was decomposed in the usual manner with the aid of alkali and barium chloride and the protein hormone adsorbed from solution by addition of a concentrated solution of benzoic acid in alcohol Removal of benzoic acid from the precipitate by washing with water and acetone gave a colorless powder with a high degree of activity From each kilogram of acetone-dried anterior pituitary powder 15 grams of the product were obtained This yield was in good agreement with that just described for acetic acid extraction and picric acid precipitation Flavianic acid has also been employed as a precipitant of thyrotrophin and was reported (16) to remove quantitatively the hormone from solution

Salt fractionation of pituitary extracts has yielded active thyrotrophic hormone preparations Fevold, Lee, Hisaw and Cohn (64) extracted whole beef pituitary tissue with dilute ammonia at pH 8 0 and removed insoluble material precipitated by addition of ammonium sulfate to 0.28 molar concentration Acidification to pH 5 4 yielded another precipitate, the supernatant was then fractionated with further careful addition of the inorganic salt at pH 7 0 Thyrotrophic activity was largely precipitated at 3 4 molar concentration of ammonium sulfate The yield of final product was poor as compared to that of other procedures in the literature Salt fractionation has also been applied (73) with considerably greater success to dilute acetic acid extracts of acetone-dried pituitary powder The hormone was precipitated by addition of ammonium sulfate to 0 6 saturation, after previously removing inactive material at 0.3 saturation Repetition of this procedure yielded a product assaying 170 chick units per milligram of nitrogen Solution of the product in water, addition of acetone to 39 per cent concentration, removal of the precipitate which formed, and addition to the supernatant of 8 to 10 volumes of acetone gave a fraction assaying 480 chick units per milligram of nitrogen The yield from a kilogram of dried pituitary powder was 2.8 grams (corrected for ash and moisture) of material assaying 60 chick weight units per milligram of product This product could be purified further by solution in water, followed by precipitation of inert protein with one volume of alcohol, and finally by precipitation of thyrotrophic activity with an excess of acetone This product assayed about 1000 chick units per milligram of nitrogen However, the total recovery of potency in this final step was low, even when adequate precautions were taken regarding temperature and length of exposure to organic solvents Junkmann (87) and other investigators have also reported losses of thyrotrophic potency in purification procedures and these losses have generally been attributed to the deleterious action of the organic solvents employed However, the significant solubility of thyrotrophin in high concentrations of alcohol and acetone might be a more important basis for the lower yields encountered

An extensive investigation of methods for the purification of thyrotrophic hormone was reported in 1941 by Jorgensen and Wade (86) These investigators evaluated various solvents for the extraction of thyrotrophin and concluded

that 0 02 normal barium hydroxide was most efficient From crude extracts it was possible to precipitate 60 to 90 per cent of the active factor, together with most of the other proteins, with flavianic, trichloroacetic, picric, phosphotungstic and sulfosalicylic acids However, with more highly purified preparations of the hormone, it was reported that these reagents yielded little or no precipitate, and that the thyrotrophin remained in solution These observations were difficult to assess inasmuch as concentrations of reagents and of protein solutions were not given The hormone was readily adsorbed from solution by a variety of agents but was difficult to elute Permutit adsorption and elution from the permutit proved the most satisfactory of the procedures tested Optimum adsorption occurred at pH 4 5 but required large quantities of permutit Elution of activity was obtained by addition of sodium hydroxide to pH 13 0 Repetition of this process proved unsatisfactory, apparently, certain impurities favored adsorption of thyrotrophin on permutit Additional efforts to achieve further purification of the hormone culminated in the use of uranium acetate as a precipitant, with recovery of the activity from the precipitate by extraction with dilute phosphate solution The degree of purification achieved by Jorgensen and Wade cannot be evaluated from their data which stress recovery of hormone with no indication of potency of preparations in terms of dry weight or nitrogen content

Divergent results were obtained in attempts to purify thyrotrophin with the aid of the ultracentrifuge No detectable sedimentation of thyrotrophic activity was observed (146) in 3 hours' ultracentrifugation at 39,000 revolutions per minute, as judged by bioassay of the top, middle and the lower portions of the contents of the centrifuge tubes In later experiments (90), both thyrotrophic and gonadotrophic activity were observed to sediment at approximately the same rate and in proportion to the rate of sedimentation of other proteins in the crude extracts employed It is not unlikely that there may have occurred adsorption of physiologically active principles on contaminating proteins

Recently, Ciereszko (32) has described a relatively simple procedure for the preparation of highly purified thyrotrophin from either whole beef or sheep pituitary glands The course of progress of this study had been indicated in abstracts published by White and Ciereszko (169) in 1941, and by the same investigators in 1942 (33) In a previous publication from this same laboratory, Bonsnes and White (22) had prepared a thyrotrophin-rich product by using isoelectric and acetone precipitations for the fractionation of saline extracts of fresh beef pituitary glands Following removal of other proteins by adjustment of hydrogen ion concentration and the use of increasing concentrations of acetone, thyrotrophic activity was found to be largely concentrated in a fraction precipitated by 75 per cent acetone at pH 4 0 Ciereszko (32) achieved further purification by making use of the previously discussed evidence that thyrotrophin is water soluble and not readily precipitated from solution by certain of the common protein precipitants Extraction of the 75 per cent acetone insoluble precipitate with water was followed by removal of protein impurities from the extract by the successive use of lead acetate and trichloroacetic acid From the

supernatant, a product was obtained with a minimum effective dose (histological chick thyroid assay) of one microgram One kilogram of glands yielded about 500 milligrams of final product The isolated material accounted for approximately 80 per cent of the thyrotrophic potency of the original saline extract of the glands Preliminary studies of the beef thyrotrophin in the Tiselius apparatus and the ultracentrifuge indicated that the product was homogeneous Biological evidence of the absence of prolactin, gonadotrophic or growth hormone activity was obtained It is interesting to note that the application of a similar procedure for purification of thyrotrophin to sheep pituitary glands yielded a final product which was not homogeneous in the Tiselius apparatus Bioassay revealed the presence of gonadotrophic hormone in these preparations The relatively lower content of gonadotrophins in the anterior pituitary gland of cattle, as compared to sheep, aids in circumventing the somewhat difficult detail of separating thyrotrophic from gonadotrophic activity

*Properties of thyrotrophin* Most of the data for the physical and chemical properties of thyrotrophin have been obtained with impure products Some of the chemical properties have been inferred from behavior of the active material in the course of its purification Consequently, the published physical and chemical behavior of the hormone may have been influenced by the presence of contaminating substances

Opinions regarding the solubility of the hormone appear to be in good agreement. It is soluble in water over the entire pH scale, and is insoluble in alcohol, ether, pyridine, acetone, methanol, chloroform and hexachloroethane (115) The active material is soluble in aqueous alcohol (73) aqueous acetone (22, 32, 73) and aqueous pyridine (115, 139) The hormone is not precipitated by dilute solutions of sulfosalicylic acid (88), trichloroacetic acid (32) or lead acetate (32) Thyrotrophin is precipitated from aqueous solution by flavianic (16, 86), picric (86, 87), tungstic (88), or phosphotungstic acids (32, 86), and by uranium acetate (86) and mercuric chloride (32) Thyrotrophin has also been reported to be precipitated by trichloroacetic or sulfosalicylic acids (86), in disagreement with the observations just cited, but the conditions under which precipitation occurred were not described

The thyrotrophic hormone is very readily adsorbed on a variety of materials, e.g., permutit (86), benzoic acid (88), colloidal ferric hydroxide (88), animal charcoal (86), and various protein precipitants (86)

Purified thyrotrophin (32) gave the usual protein color reactions, and the Molisch reaction was positive Qualitative tests for phosphorus were negative The nitrogen content has been reported as 13 per cent (73) and as 12 6 per cent (32), and two laboratories (14, 73) have reported the presence of glucosamine in thyrotrophin preparations The hormone was heat labile both in the presence or absence of oxygen (88 115), and was not active when administered orally or after digestion by proteolytic enzymes (30) These observations both confirm the protein nature of the hormone and suggest that the physiological activity is dependent upon the protein molecule as a whole

Loesser (115), working with an impure preparation of thyrotrophic hormone,

observed that the activity was destroyed by treatment with benzoyl chloride or nitrous acid Cysteine treatment was found (73) to inactivate the thyrotrophic hormone, presumably because of a reduction of disulfide linkages in the protein hormone Brief treatment of a solution of thyrotrophic hormone with ketene also produced a significant degree of inactivation (73)

Although further work must be done to characterize fully the thyrotrophic hormone, it seems clear that it is a protein and that its physiological activity is closely related to the integrity of the protein molecule A relatively small molecular weight for thyrotrophin is indicated by its slow rate of sedimentation in the ultracentrifuge, its failure to precipitate with protein precipitants like trichloroacetic acid and lead acetate, and the high concentration of inorganic salts or of organic solvents required to precipitate the active material from aqueous solution Evidence has been obtained for the presence of a carbohydrate grouping in thyrotrophin

*The Growth Hormone* The first demonstration of the capacity of anterior pituitary extracts to promote accelerated growth in mammals was made by Evans and Long (50) in 1921 Beef anterior pituitary tissue was ground in a mortar with sand and Locke's solution The clarified extract was administered daily intraperitoneally to young rats and produced a markedly increased rate of weight gain as compared to uninjected controls Later, Smith (155) found that anterior pituitary extracts could cause the resumption of growth of hypophysectomized rats The demonstration of growth-promoting activity in anterior pituitary tissues extracts led to the conclusion that the pituitary gland secreted a hormonal principle which was termed "growth hormone "

*Methods of assay* A hormone which has a stimulating effect on growth might be expected to influence the rate of a variety of chemical and physiological processes in the organism Consequently, a number of procedures have been devised for assay of the anterior pituitary growth hormone The most widely employed and reliable methods are (a) the gain in weight produced in plateaued female rats, and (b) the resumption of growth and subsequent gain in weight of hypophysectomized rats A relatively recent method of growth hormone assay has been described by Evans and his colleagues (54) This method is based on the fact that hypophysectomy causes regressive changes at the proximal end of the tibia in the immature rat These changes can be reversed with growth hormone, the increase in width of the cartilage observed during the administration of hormone is employed as the criterion of assay The bone [illegible] is stated to be approximately three times as sensitive as body weight methods [illegible] basis of daily dose, and requires one-fourth as much time and approxima[illegible]e tenth of the quantity of hormone necessary for a corresponding respo body weight method Other bioassay procedures, less often employ also been described (166)

*Preparation of growth hormone* Early in the chemical efforts to anterior pituitary growth hormone, it was observed that the biological diminished in strongly acid media Consequently, alkaline extractio pituitary tissue has generally been employed as an initial step Solut

sodium hydroxide, ammonium hydroxide, barium hydroxide or calcium hydroxide will extract the growth promoting principle from pituitary tissue Weakly alkaline, saline extracts have also proven useful as starting materials for fractionation of growth hormone activity (22, 64) Unfortunately, these alkaline solvents are also effective in extracting all other types of hormonal activity, as well as non-hormonal proteins Consequently, the subsequent purification and concentration of growth hormone has proven relatively difficult as compared to the preparation in purified form of the other anterior pituitary hormones Barium and calcium hydroxides have been most frequently used as extraction media, neutralization and filtration of the extract yielded a solution which exhibited growth promoting potency (47) Gonadotrophic activity could be removed almost completely from the growth principle by repeated fractionation with 20 per cent sodium sulfate (163) The precipitated proteins contained growth hormone but were relatively free of the gonadotrophic factors

The use of adsorbents for removal of growth hormone from extracts has been extensively studied Whereas aluminum hydroxide and Lloyd's reagent were not effective adsorbing agents, some success has been reported with calcium phosphate and norite. Collip, Selye and Thomson (38) adsorbed the growth factor on calcium phosphate and eluted with dilute alkali A growth preparation was obtained which was reported to be relatively free of the thyrotrophic adrenotrophic, and gonadotrophic hormones as well as prolactin and the metabolic factors (ketogenic and diabetogenic) Dingemanse (42) and Dingemanse and Freud (43) have removed growth hormone from alkaline extracts of acetone-dried pituitary glands by adsorption on norite The hormone was eluted with liquid phenol and obtained as a precipitate when the phenol solution was poured into alcohol-ether mixture The properties of the isolated product were studied in detail. Its elementary composition was that of a protein with a total sulfur content of 1 47 per cent, this was present as disulfide groups The hormonal activity was destroyed by heat, by strong acid or alkali and by proteolytic enzymes The hormone was reported to be insoluble at pH 8 0 and could be ultrafiltered or dialyzed at pH 10 0 Assayed in hypophysectomized rats, the product stimulated a weight gain of one gram a day for seven days when given in doses of ten micrograms daily At a dose level of 30 micrograms daily the product showed no lactogenic, thyrotrophic, gonadotrophic, or adrenotrophic activity Although certain of the claims of Dingemanse have not been fully substantiated by other investigators (46, 142), it is interesting to note certain similarities in properties of her product with those reported for the highly purified growth hormone (see below)

Investigators in the laboratory of H M Evans at the University of California have been persistently pursuing the task of purification and isolation of the growth hormone since the first description of this principle in that laboratory twenty-five years ago It is wholly fitting that these efforts have recently been crowned with final and apparently complete success An earlier study by Evans, Meyer and Simpson (51) examined a wide variety of extraction, fractionation and purification procedures for the preparation of growth hormone Dilute

barium or calcium hydroxide appeared to be the solvents of choice Growth-promoting activity was found to be in the globulin fraction precipitated from alkaline extracts by half saturation with ammonium sulfate (56) Many methods were explored in efforts to free this precipitate of other types of hormonal activity Reprecipitation of the globulin fraction, extraction of the gonadotrophic activity with 10 per cent saline, removal of the lactogenic hormone by precipitation with bromine water or by extraction with 0 1 saturated ammonium sulfate solution, were all attended by varying degrees of success but with no great increase in growth hormone potency While biological purification may have been obtained by these procedures, little chemical purification was accomplished At a later time (69, 123) considerable advance was reported from the California laboratory in the purification of the growth hormone by cysteine treatment of the ammonium sulfate-insoluble, growth hormone globulin fraction Alkaline cysteine solutions were added to alkaline (pH 8 5) solutions of the globulin fraction which contained growth hormone activity, the mixtures were allowed to stand at room temperature for one to two days Under these conditions, protein impurities precipitated from the solution with a resulting purification and concentration of growth hormone activity Although the best product obtained by application of the cysteine procedure showed two components in the Tiselius apparatus, only five micrograms daily were reported required to produce a body weight increase of one gram per day in hypophysectomized rats Moreover, the final product was so low in physiologically active contaminants that a total dose of ten milligrams did not cause any demonstrable stimulation of ovaries, thyroids, or adrenals in hypophysectomized rats

The isolation of a protein with growth hormone activity and showing electrophoretic homogeneity was reported in a preliminary note by Li and Evans (95) in 1944, and more recently this work has been described in detail by Li, Evans and Simpson (97) The authors have dropped the cysteine procedure and relied wholly on the application of salt fractionation and isoelectric precipitation to alkaline extracts of acetone-dried beef anterior pituitary powder The growth-promoting, crude globulin fraction was again isolated from an alkaline extract of acetone-dried beef pituitary powder by a procedure essentially that described previously (56) The globulin precipitate was then dissolved and freed of a protein insoluble at 0 6 molar concentration of ammonium sulfate After precipitation of growth-promoting activity by increasing the salt concentration to 2 0 molar, further purification was achieved by fractionation with sodium chloride The globulin fraction was dissolved by the addition of dilute hydrochloric acid until the pH was 4 0 Saturated sodium chloride solution was then added to a salt concentration of 0 10 molar, a precipitate formed which was devoid of growth activity and therefore was discarded Addition of solid sodium chloride to the supernatant to a concentration of 5 0 molar precipitated growth hormone activity This sodium chloride fractionation was repeated twice on the precipitate obtained at this stage The final 5 0 molar sodium chloride precipitate was dissolved in water and dialyzed until free of salt This solution was then adjusted to pH 5 7 to 5 8 and the precipitate discarded Another precipi-

tate was removed at pH 8 7 to 8.8 and the clear supernatant brought to pH 7.0 Addition of ammonium sulfate to 1 65 molar concentration precipitated growth hormone activity The pH alterations and ammonium sulfate fraction ation were repeated twice The dialyzed solution of the final 1 65 molar ammonium sulfate precipitate was precipitated again at pH 5 7 to 5.8 and pH 8 7 to 8.8 as above and finally at pH 6.8 to 6 9 in the absence of salt This isoelectric precipitation was repeated twice The final precipitate obtained at pH 6.8 to 6.9 was the purified growth hormone

A final yield of approximately 40 mgm of the growth hormone was obtained from one kilogram of fresh, beef anterior pituitary glands In this respect the yield of growth hormone is lower than that reported for the other anterior pituitary hormones and may be in part a reflection of the lengthy procedure necessary to obtain the final product The isolated protein was shown to be homogeneous in electrophoretic, dialysis and solubility studies In addition, no evidence could be obtained for the presence of any of the other known pituitary hormones The hypophysectomized rat was used to establish the absence of adrenotrophic, thyrotrophic and gonadotrophic hormones, and the absence of prolactin was demonstrated by the pigeon assay The absence of thyrotrophic hormone might have been demonstrated more conclusively by employing the highly sensitive chick assay method, since the rat thyroid is relatively non-responsive to small amounts of thyrotrophic hormone Thyrotrophic activity is one of the most common contaminants of various pituitary fractions because of the high degree of adsorption of thyrotrophin on protein precipitates

The isolated growth hormone produced a daily body weight increase of one gram when injected in a dose of ten micrograms daily for ten days in hypophysectomized rats A similar daily dose level for four days was sufficient to increase the width of the uncalcified portion of the proximal epiphyseal cartilage of the tibia 50 per cent over that of the control The protein had an isoelectric point at pH 6.85 and osmotic pressure measurements indicated a molecular weight of 44,250 The hormonal activity was destroyed by incubation of the protein with pepsin or trypsin The protein was unstable at the temperature of boiling water and destruction of activity ensued. In buffer of pH 7 0, the protein hormone was coagulated at 70 to 80 degrees centigrade, with accompanying destruction of growth promoting potency The protein exhibited greater stability in alkaline than in acid medium, and its biological activity was unaffected by concentrated urea solutions Elemental analysis revealed a composition characteristic of proteins, with a sulfur content of 1.30 per cent Cysteine was absent from the molecule In a recent abstract, Li (94) has reported that the growth hormone contains 3 06 per cent methionine and 2.25 per cent cystine These values for the two sulfur-containing amino acids satisfactorily accounted for the total sulfur of the protein Other amino acid analyses of the growth hormone protein (97) revealed 4.30 per cent tyrosine, 0 02 per cent tryptophane and 13 40 per cent glutamic acid in the molecule

*The Gonadotrophic Hormones* The marked atrophy of the occurs following removal of the anterior pituitary gland early and

relationships between the pituitary body and the gonads These relationships were emphasized twenty years ago by the demonstration in two laboratories (154, 172) that precocious sexual maturity could be produced in immature mice and rats by the implantation of either the whole pituitary gland or its anterior lobe At the present time it is well established that three gonadotrophic hormones are secreted by the anterior pituitary gland These are the follicle-stimulating hormone which causes the growth of Graafian follicles preparatory to the release of ova from the female gonad and stimulates the sperm-forming tissue of the testes, the luteinizing (or interstitial cell-stimulating) hormone, which stimulates the interstitial cells of the ovaries or testes and causes the formation of corpora lutea from preformed Graafian follicles, and prolactin, which has been shown to have a significant rôle in the regulation of the functional activity of the corpus luteum (52, 53, 55) Prolactin has been discussed previously in this review

The debate in the early literature regarding the unity or the multiplicity of the pituitary gonadotrophic complex was greatly clarified in 1931 when Fevold, Hisaw and Leonard (63) succeeded by chemical fractionation in separating a follicle-stimulating fraction of pituitary extracts from one rich in luteinizing substance This work was subsequently confirmed and extended, and culminated in the isolation in highly purified form of two chemically distinct protein preparations, one of which has the physiological properties of the follicle-stimulating hormone, the other of the luteinizing hormone (28, 29, 58, 60) Coffin and van Dyke (34) have proposed the etymologically justified names, derived from Greek roots, of thylakentrin for the follicle-stimulating hormone and metakentrin for the interstitial cell-stimulating, or luteinizing hormone

*Methods of assay* The follicle-stimulating hormone induces the growth of a large number of Graafian follicles, resulting in a gross increase in weight of the ovaries This forms the basis of a sensitive and highly specific assay method in the hypophysectomized animal Other assay methods are of unquestionable validity only in the absence of luteinizing hormone The latter hormone may be assayed on the basis of its capacity to increase the weight of the ovaries of immature female rats when administered together with follicle-stimulating hormone, or by its capacity to increase the weight of any one or all of the secondary sex glands of the immature or hypophysectomized male rat

*Preparation of the follicle-stimulating hormone* Although preparations of follicle-stimulating hormone have been obtained which are free of almost all traces of luteinizing factor as well as of other pituitary hormones, no single product has as yet been isolated which has only thylakentrin activity and satisfies all of the usual criteria of protein purity Most of the methods described for the purification of follicle-stimulating hormone have been chiefly concerned with the problem of freeing the preparations of the luteinizing substance The chief types of procedures employed will be briefly reviewed

Sheep and hog pituitary glands have been the chief starting material for the preparation of follicle-stimulating hormone Human or horse pituitaries would be more logical sources if available in adequate supply, since the pituitary glands in these species are relatively rich in follicle-stimulating principle and poor in

luteinizing hormone Gonadotrophic activity is readily extracted from the fresh tissue or from acetone-desiccated pituitary powder by alkaline aqueous solvents Fevold and his colleagues (57, 58, 61, 62, 63) generally employed aqueous pyridine to extract acetone-dried pituitary glands, although any aqueous solvent could be employed if the pH was more alkaline than 6 0 Extraction with water also gave an extract with gonadotrophic activity (129) The various extracts have been subsequently fractionated in several ways in efforts to separate the two gonadotrophic principles Evans and his colleagues (49) have taken advantage of the greater solubility of thylakentrin in ammonium sulfate solution Other investigators (84, 85, 138) have employed a similar approach Fraenkel-Conrat, Simpson and Evans (71) obtained a preparation of follicle-stimulating hormone which on preliminary electrophoretic examination contained chiefly one component However, no evidence was presented that the main component was follicle-stimulating substance and, although their preparations were highly active in this principle, they still contained some luteinizing hormone

Fevold (59) also reported the preparation of highly purified follicle-stimulating hormone by using ammonium sulfate or acetone fractionation Advantage was taken of the relative solubility of the hormone in 45 per cent acetone at pH 4.2, under these conditions the luteinizing hormone exhibited limited solubility The most highly purified preparation of thylakentrin obtained by Fevold also contained contaminating metakentrin A considerable degree of separation of thylakentrin from metakentrin has also been achieved (164) by fractionation with alcohol at −6 degrees centigrade At this temperature the luteinizing principle was insoluble at 40 per cent alcohol concentration

In 1938 McShan and Meyer (126) made the interesting observation that tryptic digestion had a destructive action on the luteinizing factor but left follicle-stimulating activity unimpaired This observation was also made independently by Chen and Van Dyke (26) Although this difference in stability of the two gonadotrophic hormones toward tryptic digestion was not confirmed by Abramowitz and Hisaw (1), subsequent study by Chow, Greep and Van Dyke (30) revealed that the basis for the lack of agreement between the two laboratories was the degree to which tryptic digestion had proceeded Thus, when crude hog pituitary extract was digested with trypsin, follicle-stimulating activity still remained when proteolysis had not exceeded 48 per cent of that theoretically possible At this point, luteinizing activity was completely destroyed However, when the extent of digestion was greater, follicle-stimulating potency was also abolished These data have been confirmed by McShan and Meyer (128) who utilized this information to convert a crude extract, containing both gonadotrophins, into a product having only follicle-stimulating activity Further application of tryptic digestion to the problem of purification of the follicle-stimulating hormone has been described recently by the same authors (130) However, not all of the follicle-stimulating preparations obtained were entirely devoid of luteinizing activity The newer, potent preparations had been freed of a fraction which had caused reactions in the human at the site of injection (41)

hormone has been accomplished by Greep, Van Dyke and Chow (75, 77) investigators found that hog metakentrin is extremely insoluble in a s consisting of one-fourth molar acetate buffer (pH 4 4) and 20 5 per cent s sulfate, whereas thylakentrin is very soluble in this solvent The final pr proved free of luteinizing activity even when injected in hypophysectomize in amounts twenty-five times the minimum effective dose necessary to pr evidence of follicle stimulation Absence of metakentrin was also demons in hypophysectomized rats by the use of the sensitive prostate method Moreover, the presence of metakentrin could not be detected even whe purified thylakentrin preparations were tested immunochemically aga rabbit antiserum to metakentrin (27) Tests for other pituitary principle lactogenic, thyrotrophic, adrenotrophic and posterior lobe hormones gav formly negative results In view of the apparent biological purity of the fo stimulating preparations, it was particularly disappointing to find by el phoretic and ultracentrifugal analyses that the products were inhomogeneou polydisperse (28) In the separation cell of the Tiselius apparatus it was po to isolate several fractions, representing mixtures of components Examin of the distribution of follicle-stimulating activity in studies of this type indi that the isoelectric point of the follicle-stimulating hormone was at abou 4 8

*Other properties of purified thylakentrin* The solubility behavior, re stability toward trypsin, and the probable isoelectric point of the follicle-s lating hormone have been described in the previous section Other prop of some of the highly purified products need only be mentioned since they been obtained on chemically impure preparations

The suggestion that thylakentrin is probably a *glycoprotein* is based o observation (126, 127) that commercial preparations of ptyalin or takadia destroyed the follicle-stimulating but not the luteinizing activity of pitu extracts The carbohydrate could not be removed by dialysis or che fractionation Evans and his associates (48) have reported 10 to 13 per carbohydrate and 8 per cent hexosamine in their follicle-stimulating fraction sheep tissue Gurin (78) found 4 5 per cent mannose and 4 4 per cent hexosa in a preparation of swine follicle-stimulating hormone

Data on the behavior of follicle-stimulating hormone on treatment ketene, cysteine and formalin have not been in consistent agreement and repetition under careful conditions when the hormone is available in pure Reaction of the hormone with ketene at pH 5 0 for 30 minutes (107) or treat with cysteine at pH 7 7 for 48 hours (70, 128) have been reported to resu inactivation of thylakentrin

*Purification of the luteinizing hormone* The luteinizing hormone is pr in the pituitaries of all animals which have been studied As in the case o other pituitary hormones, there appears to be a species difference in the qua of luteinizing hormone present in the hypophysis Sheep pituitary tissue good starting material for purification studies because of its relatively high tent of luteinizing principle As will be indicated below, there appear to

interesting chemical differences among
various species

Preparations of lutemizing hormone
punty were isolated practically simultaneously
the pituitary tissue of sheep and swine by
Dyke and his collaborators (29, 30, 31,
with 2 per cent sodium chloride solu
adjusted to pH 4.2 to 4 6 and the pre
supernatant with ammonium sulfate ga
of salt The water insoluble precipitate
protein Half-saturation of the remaining
pH 4.2 yielded further inactive materia
then precipitated by increasing the salt
ammonium sulfate After removal of
precipitated by addition of one volume
volumes of 4 per cent sodium sulfate solu
of metakentrin from thylakentrin, the la
conditions The precipitate was dialy
lizing principle was then precipitated fr
ammonium sulfate to one-third saturation
in water and the active principle precip
equal volume of saturated ammonium
sulfate fractionation a number of time
electrophoretically and ultracentrifugally
indicated that the isolated material was
separate the biological activity from the
furnish any evidence for the presence
homogeneous protein The isolated pro
order of biological activity, 6 7 microgra
the stimulation of the ventral prostate in

Li, Simpson and Evans (108) employe
extraction of acetone-desiccated sheep pi
tion was then increased to 80 per cent an
acetic acid. The precipitated material w
After adjusting to pH 4.5 with acetic
with acetone The precipitate was
activity was present in the extract
fractionation steps using varying
metakentrin being separated at between
sulfate Separation from traces of thy
of the lutemizing principle from a 1 per
acetic acid to a concentration of 2 5 per
the final product was free of all other
trophin, growth, thyrotrophin and
chemic studies supported the suggestion

rried to the point where
wed the presence of two
horetic separation of the
ns present in the fraction
otein present

It has been previously
mone is relatively slowly
ivity is rapidly destroyed
hand, lutemizing hormone
onditions which result in
27) Other inactivation
utemizing fractions need
om these earlier studies
ctivated by ketene (107)
oward formalin has been
4) a selective destruction
ther (124) was unable to

teins On the basis of
recognized individual
among certain of the
mones in the anterior
he protein hormones
ate as judged by the
ability techniques,
biological assays
table by physico-
ituitary proteins
izing hormones
m, but has not
cle-stimulating

erior pituitary
he methods of
to purify these
in chemistry In
to be intimately
ures
of

A later report (112) described an alternative method of preparation and a physical-chemical study of the luteinizing principle Evidence for the homogeneity of the isolated protein was obtained from detailed electrophoretic examination, and from ultracentrifuge and solubility studies Osmotic pressure measurements indicated a molecular weight of 40,000 for the protein hormone The purified luteinizing hormone contained (108) 100 to 200 rat units per milligram, the unit being based on the repair of the interstitial tissue of the ovaries of hypophysectomized rats

Table 4 contains some of the published data permitting a comparison of the physical and chemical properties of metakentrin prepared from hog and from sheep pituitary glands The present evidence indicates that the luteinizing activity is associated in the two species with chemically different pituitary

TABLE 4

*Comparison of the properties of luteinizing hormone preparations from hog and from sheep pituitary glands*

| | HOG | SHEEP |
|---|---|---|
| $S_{20}$ | 6 80 S (31)* | 3 6 S (108, 112) |
| Estimated molecular weight | 100,000 (31) | 40,000 (108, 112) |
| Isoelectric point, pH | 7 45 (31) | 4 6 (108, 112) |
| Tyrosine content | | 4 5% (109) |
| Tryptophane content | 3 8% (112) | 1 0% (109) |
| Carbohydrate content | 2 0% (112) | 4 5% (112) |
| Mannose content | 2 8% (78) | 4 5% (108) |
| Hexosamine content | 2 2% (78) | 5 9% (108) |

* The numbers in parentheses are bibliography references

proteins This conclusion is supported by the demonstration (27) that hog and sheep metakentrins are immunologically distinct Rabbit antiserum prepared by injection of hog metakentrin reacted specifically with its homologous antigen, but not with sheep metakentrin or with any of the other known pituitary hormones

The Harvard laboratories have also conducted extensive studies of the purification of luteinizing hormone as part of an investigation designed to study the purification of all of the anterior pituitary protein hormones These investigations were interrupted by the advent of the war However, a significant degree of success was achieved in the concentration of several of the active principles of the pituitary, including the luteinizing hormone The initial publication (64) has already been referred to in the discussion of the purification of thyrotrophic hormone The latter hormone was found distributed between two fractions, one of which was chiefly follicle-stimulating and the other luteinizing The thyrotrophic hormone is more soluble in ammonium sulfate than is the luteinizing principle and this information was used in further purification of metakentrin (60) Following removal of thyrotrophin, the luteinizing hormone could be concentrated in fractions precipitated between 1 6 and 1 8 molar concentrations

of ammonium sulfate Further purification was carried to the point where electrophoretic and ultracentrifugal examination showed the presence of two main components in the final preparation Electrophoretic separation of the two components suggested that luteinizing activity was present in the fraction representing approximately 75 per cent of the total protein present

*Effect of various agents on the luteinizing hormone* It has been previously pointed out that whereas the follicle-stimulating hormone is relatively slowly inactivated by digestion with trypsin, luteinizing activity is rapidly destroyed by this proteolytic enzyme (26, 30, 126) On the other hand, luteinizing hormone is not inactivated by pytalin or takadiastase under conditions which result in destruction of follicle-stimulating activity (126, 127) Other inactivation studies which have been conducted with impure luteinizing fractions need repetition with the purified hormone The data from these earlier studies indicated that the luteinizing hormone was rapidly inactivated by ketene (107) and cysteine (70, 128) The stability of the hormone toward formalin has been studied in two laboratories (124, 164), one reported (164) a selective destruction of the luteinizing principle by this reagent, while the other (124) was unable to confirm this observation

## CONCLUDING REMARKS

The hormones of the anterior pituitary gland are proteins On the basis of the physiological evidence, there appear to be at least six recognized individual hormones, although there is some biological over lapping among certain of the anterior pituitary secretions The existence of other hormones in the anterior pituitary gland is not yet completely excluded Four of the protein hormones have been isolated in a homogeneous and highly purified state as judged by the application of electrophoretic, ultracentrifugal and solubility techniques, although the use of other criteria of establishing purity, e.g , biological assays and immunological behavior, may reveal impurities not detectable by physicochemical methods The four most highly purified anterior pituitary proteins are the lactogenic, the adrenotrophic, the growth and the luteinizing hormones The thyrotrophic principle has been isolated in highly purified form, but has not yet been examined by rigid criteria of protein purity The follicle-stimulating hormone awaits further purification

The isolation and characterization of the hormones of the anterior pituitary gland are problems of protein chemistry and have involved the methods of protein study The large degree of success attending efforts to purify these hormones has been related to advances in knowledge of protein chemistry In the case of most of the hormones, the biological activity seems to be intimately related to the protein nature of the active principle, and procedures which alter even slightly the protein structure result in a partial or complete loss of physiological characteristics A notable exception appears to be the adrenotrophic hormone, since a significant number of peptide linkages of this protein have been cleaved by pepsin with a reported retention of biological activity

The physiological responses produced by the anterior pituitary hormones

cannot as yet be attributed to any specific portion of the various protein molecules Efforts to detect in the protein hormones non-amino acid groups or active groupings of amino acids to which hormonal function could be assigned have been unsuccessful At least three of the active principles, i e , thet hyrotrophic, the follicle-stimulating and the luteinizing hormones, contain carbohydrate in their structures Carbohydrate-splitting enzymes inactivate the follicle-stimulating principle, and it has therefore been assumed that carbohydrate plays an important rôle in the biological activity of this hormone Definite conclusions on this point must await repetition of the experiments with purified enzymes and more highly purified hormone Of considerable significance to the problem of the relation of the structure of the protein hormones to their biological activity is the recent demonstration by Price, Cori and Colowick (136) of the *in vitro* inhibition of hexokinase by crude anterior pituitary extracts This important preliminary demonstration of the effect of an anterior pituitary hormone *in vitro* may lead to a test system in which, by the use of both purified protein hormones and of models of known structure, an approach would be possible to the problem of the relation of protein structure to hormonal action

Certain physical and chemical differences exist among anterior pituitary hormones isolated from different species These variations are most striking in a comparison of the data for the luteinizing hormone preparations obtained from hog and sheep pituitary tissue Preliminary data (166) also suggest that the thyrotrophic hormone from beef glands may not be the same protein as the hormone from sheep pituitaries Less striking differences are seen between the lactogenic hormones from beef and sheep glands, since variations only in tyrosine content and solubility behavior have been observed On the other hand, the purified hog and sheep adrenotrophins, isolated by two widely different procedures, appear to be identical in every property which has been examined

## REFERENCES

(1) Abramowitz, A A and F L Hisaw Endocrinology 25 633, 1939
(2) Adler, L Arch Entw Mech Org 39 21, 1914
(3) Allen, B M Science 44 755, 1916
(4) Anderson, E M and J B Collip J Physiol 82 11, 1934
(5) Anselmino, K J , F Hoffmann and L Herold Klin Wchnschr 13 209, 1934
(6) Aron, M Compt rend soc biol 102 682, 1929
(7) Aschner, B Pflüger's Arch 146 1, 1912
(8) Ascoli, G and T Legnani Boll Soc med chir Pavia 24 1911
(9) Astwood, E B Endocrinology 28 309, 1941
(10) Astwood, E B and R Tyslowitz Fed Proc 1 pt 2, 4, 1942
(11) Bates, R W Cold Spring Harbor Symposia on Quantitative Biology 5 191, 1937
(12) Bates, R W Personal communication
(13) Bates, R W and O Riddle J Pharmacol and Exper Therap 55 365, 1935
(14) Bates, R W , O Riddle and R A Miller Endocrinology 27 781, 1940
(15) Bauer, H and E Strauss Ber Chem Ges 68 1108, 1935
(16) Bergman, A J , O B Houchin and C W Turner Endocrinology 25 547, 1939
(17) Bergman, A J and C W Turner J Biol Chem 118 247, 1937
(18) Bergmann, M and F Stern Ber Chem Ges 63 437, 1930
(19) Bischoff, H W and W R Lyons Endocrinology 25 17, 1939

(20) Bomskov, C Methodik der Hormonforschung I, 922 1937 G Thieme Leipzig.
(21) Bomskov, C and L Sladovic Ztschr physiol Chem 264: 274 1940
(22) Bonanes, R W and A White Endocrinology 26: 990 1940
(23) Bottomley A C and S J Folley Nature 145 304, 1940
(24) Brand, E Private communication
(25) Brand, E and B Kassell J Biol Chem 131 489, 1939
(26) Chen, G and H B Van Dyke Proc Soc Exper Biol and Med 40: 172, 1939
(27) Chow, B F Endocrinology 30: 657, 1942
(28) Chow, B F Ann N Y Acad Sci 43: 309, 1943
(29) Chow, B F Advances in protein chemistry I, 153 1944 Academic Press, N Y
(30) Chow B F, R. O Greep and H B Van Dyke J Endocrinology 1: 440, 1939
(31) Chow, B F, H B Van Dyke R O Greep A Rothen and T Shedlovsky Endocrinology 30 650 1942
(32) Ciereszko, L S J Biol Chem 160: 585 1945
(33) Ciereszko L S and A White Fed Proc 1: 105 1942
(34) Coffin, H C and H B Van Dyke Science 93: 61, 1941
(35) Collip, J B J Mt Sinai Hosp 1 28 1934
(36) Collip J B J A. M A 115 2073 1940
(37) Collip, J B., E M Anderson and D L Thomson Lancet 2: 347 1933
(38) Collip J B, H Selye and D L. Thomson Proc Soc Exper Biol and Med. 30: 544, 1932-33
(39) Corner G W, Sr Endocrinology 33 405 1943
(40) Coulter, C B, F N Stone and E A Kabat J Gen. Physiol 19: 739, 1935-36
(41) Davis, M E and A A Hellbaum J Clin Endocrinology 4: 400, 1944
(42) Dingemanse E Kongressbericht des XVI Internationalen Physiologen Kongress Free Vereinegung Schweiz Physiologen Zurich p 320
(43) Dingemanse, E and J Freud Acta Brev Neerland 5 109 1935
(44) Evans H M Harvey Lectures 19 212, 1923-24 Williams & Wilkins Co Baltimore
(45) Evans, H M J A M A 101 425 1933
(46) Evans H M Assoc Res Nervous and Mental Dis, Proc 17: 175, 1938
(47) Evans H M, R E Cornish and M E Simpson Proc Soc Exper Biol and Med 27 101, 1929-30
(48) Evans H M H L Fraenkel-Conrat M E Simpson and C H Li Science 89: 249, 1939
(49) Evans H M K. Korpi, R. F Penchaks and M E Simpson Univ of Calif Pub in Anatomy 1 237 1936
(50) Evans, H M and J A Long Anat Rec 21 62, 1921
(51) Evans, H M K. Meyer and M. E Simpson Univ Calif Memoirs 11 305 1933
(52) Evans H M M. E Simpson and W R Lyons Proc Soc Exper Biol and Med 46: 586 1941
(53) Evans H M M E Simpson W R Lyons and K Turpeinen Endocrinology 28: 933 1941
(54) Evans, H M, M E Simpson W Marx and E Kibrick Endocrinology 32: 13 1943
(55) Evans H M M E. Simpson and K. Turpeinen Anat Rec Suppl 70: 26, 1937-38
(56) Evans, H M N Uyei, Q R. Bartz and M E Simpson Endocrinology 22: 483 1938
(57) Fevold H L Cold Spring Harbor Symposia on Quantitative Biology 5 93, 1937
(58) Fevold, H L In Sex and internal secretions 2nd ed Williams & Wilkins Co., Baltimore 1939 Ed by E Allen p 956
(59) Fevold, H L Endocrinology 28: 33 1941
(60) Fevold H L Ann N Y Acad Sci 43: 321 1943
(61) Fevold H L and F L Hisaw Am J Physiol 109: 655, 1934

(62) Fevold, H L, F L Hisaw, A Hellbaum and R Hertz Am J Physiol 104 710, 1933
(63) Fevold, H L, F L Hisaw and S L Leonard Am J Physiol 97 291, 1931
(64) Fevold, H L, M Lee, F L Hisaw and E J Cohn Endocrinology 26 999, 1940
(65) Fleischer, G A J Biol Chem 147 525, 1943
(66) Folley, S J Biol Rev 15 421, 1940
(67) Folley, S J, F J Dyer and K H Coward J Endocrinology 2 179, 1940–41
(68) Fraenkel-Conrat, H J Biol Chem 142 119, 1942
(69) Fraenkel-Conrat, H L, D L Meamber, M E Simpson and H M Evans Endocrinology 27 605, 1940
(70) Fraenkel-Conrat, H, M E Simpson and H M Evans J Biol Chem 130 243, 1939
(71) Fraenkel-Conrat, H L, M E Simpson and H M Evans Proc Soc Exper Biol and Med 45 627, 1940
(72) Fraenkel-Conrat, H, M E Simpson and H M Evans J Biol Chem 142 107, 1942
(73) Fraenkel-Conrat, J, H Fraenkel-Conrat, M E Simpson and H M Evans J Biol Chem 135 199, 1940
(74) Gardner, W U and C W Turner Missouri Agric Exp Sta Research Bull no 196, 1933
(75) Greep, R O, H B Van Dyke and B F Chow J Biol Chem 133 289, 1940
(76) Greep, R O, H B Van Dyke and B F Chow Proc Soc Exper Biol and Med 46 644, 1941
(77) Greep, R O, H B Van Dyke and B F Chow Endocrinology 30 635, 1942
(78) Gurin, S Proc Soc Exper Biol and Med 49 48, 1942
(79) Herring, V V and H M Evans Am J Physiol 140 452, 1943
(80) Herriott, R M J Gen Physiol 19 283, 1935–36
(81) Herriott, R M and J H Northrop J Gen Physiol 18 35, 1934–35
(82) Heyl, J G Acta Brevia Neerland 3 111, 1933
(83) Janssen, S and A Loeser Arch exper Path u Pharmakol 163 517, 1931–32
(84) Jensen, H, M E Simpson, S Tolksdorf and H M Evans Endocrinology 25 57, 1939
(85) Jensen, H, S Tolksdorf and F Bamman J Biol Chem 135 791, 1940
(86) Jorgensen, M N and N J Wade Endocrinology 28 406, 1941
(87) Junkmann, K In Handbuch der biologischen Arbeitsmethoden, Abt V, Teil 3B 2 Halfte, p 1072, 1936
(88) Lambie, C G and V M Trikojus Biochem J 31 843, 1937
(89) Lavin, G I and J H Northrop J Am Chem Soc 57 874, 1935
(90) Levin, L, G K Smelser, D H Moore and A E Severinghaus Endocrinology 30 171, 1942
(91) Li, C H J Biol Chem 146 633, 1942
(92) Li, C H J Biol Chem 148 289, 1943
(93) Li, C H J Biol Chem 155 45, 1944
(94) Li, C H Fed Proc 5 144, 1946
(95) Li, C H and H M Evans Science 99 183, 1944
(96) Li, C H, H M Evans and M E Simpson J Biol Chem 149 413, 1943
(97) Li, C H, H M Evans and M E Simpson J Biol Chem 159 353, 1945
(98) Li, C H and A Kalman J Am Chem Soc 68 285, 1946
(99) Li, C H, W R Lyons and H M Evans J Gen Physiol 23 433, 1939–40
(100) Li, C H, W R Lyons and H M Evans J Am Chem Soc 62 2925, 1940
(101) Li, C H, W R Lyons and H M Evans J Biol Chem 136 709, 1940
(102) Li, C H, W R Lyons and H M Evans J Gen Physiol 24 303, 1940–41
(103) Li, C H, W R Lyons and H M Evans J Biol Chem 139 43, 1941
(104) Li, C H, W R Lyons and H M Evans J Biol Chem 140 43, 1941

(105) Li, C H , W R Lyons, M E Simpson and H M Evans Science 90: 376 1939
(106) Li, C H , M E Simpson and H M Evans Science 90 140, 1939
(107) Li, C H M E Simpson and H M Evans J Biol Chem 131: 259 1939
(108) Li, C H , M E Simpson and H M Evans. Endocrinology 27: 803 1940
(109) Li, C H., M E Simpson and H M Evans Science 92: 355 1940
(110) Li, C H., M E Simpson and H M Evans Science 96 450, 1942
(111) Li, C H., M E Simpson and H M. Evans J Biol Chem 146: 627, 1942
(112) Li, C H , M E. Simpson and H M Evans J Am Chem Soc 64: 367, 1942
(113) Li, C H , M. E Simpson and H. M Evans Arch Biochem 9: 259, 1946
(114) Loeb, L and R. B Bassett Proc Soc Exper Biol Med 26: 860 1928-29
(115) Loesser, A Arch exper Path u Pharmakol 168: 693 1932
(116) Lugg J W H Biochem J 32 775 1938
(117) Lyons, W R Proc Soc Exper Biol and Med 35: 645, 1936-37
(118) Lyons, W R Cold Spring Harbor Symposia on Quantitative Biology 5 198, 1937
(119) Lyons, W R. Endocrinology 28: 161, 1941
(120) Lyons W R and H R. Catchpole Proc Soc Exper Biol and Med 31: 299 1933-34
(121) Lyons W R. and E Page Proc Soc Exper Biol and Med 32: 1049, 1934-35
(122) Marx W , E Anderson, C T O Fong and H M. Evans Proc. Soc Exper Biol and Med 53: 38, 1943
(123) Marx W , M E Simpson and H M Evans J Biol Chem 147: 77, 1943
(124) Maxwell, L. C and F Bischoff J Biol Chem 112: 215 1935-36
(125) McShan, W H and H E French J Biol Chem 117: 111, 1937
(126) McShan W H and R. K Meyer J Biol Chem. 126 361, 1938
(127) McShan, W H and R. K. Meyer Proc. Soc Exper Biol and Med 40 701, 1939
(128) McShan, W H and R. K. Meyer J Biol Chem 135: 473, 1940
(129) McShan, W H and R K Meyer J Biol Chem 151 259 1943
(130) McShan W H and R. K. Meyer. Proc Soc Exper Biol and Med 61 57, 1946
(131) McShan W H and C W Turner Proc Soc Exper Biol and Med 32: 1655 1934-35
(132) Miller, G L and W M Stanley J Biol Chem 141 905 1941
(133) Moon, H D Proc Soc Exper Biol and Med 35 649 1936-37
(134) Nelson W O Physiol Rev 16 488, 1936
(135) Organon N V Chem Abs 34 8184 1940
(136) Price W H C F Cori and S P Colowick J Biol Chem 160 633, 1945
(137) Riddle O and R W Bates In Sex and internal secretions 2nd ed Williams & Wilkins Co Baltimore 1939 Ed by E Allen p 1088
(138) Rinderknecht, H and P C Williams J Endocrinology 1 117, 1939
(139) Rowlands, I W and A S Parkes Biochem J 28: 1829 1934
(140) Sayers G , A White and C N H Long Proc Soc Exper Biol Med 52: 199 (1943)
(141) Sayers G , A White and C N H Long J Biol Chem 149: 425, 1943
(142) Schooley, J P , O Riddle and R. W Bates Am J Anat 69: 123 1941
(143) Schwenk, E Personal communication.
(144) Schwenk, E , G A Fleischer and S Tolksdorf J Biol Chem 147 535, 1943
(145) Sevag, M. G Biochem Ztschr 273: 419 1934
(146) Severinghaus A E , L Levin and J A Chiles Jr Endocrinology 23: 285, 1938.
(147) Shaw, J L. D and W D McFarlane Canadian J Res B16: 361, 1938 J Biol Chem 122: 387 1940
(148) Shedlovsky T Ann N Y Acad Sci 43: 259, 1943
(149) Shedlovsky T , A Rothen R. O Greep, H B Van Dyke and B F Chow Science 92: 178 1940
(150) Shipley, R. A K G Stern and A. White J Exper Med 69: 785 1939
(151) Simpson, M E H M Evans and C H Li Endocrinology 33: 261, 1943
(152) Smith P E Anat Rec 11: 57 1916-17

(153) Smith, P E Anat Rec 32 221, 1926
(154) Smith, P E Proc Soc Exper Biol and Med 24 131, 1926–27
(155) Smith, P E J A M A 88 158, 1927
(156) Smith, P E Am J Anat 45 205, 1930
(157) Stern, K G and A White J Biol Chem 122 371 ,1937–38
(158) Stricker, P and F Grueter Compt rend Soc Biol 99 1978, 1928
(159) Thurston, E W Arch Path 15 67, 1933
(160) Turner, C W In Sex and internal secretions 2nd ed Williams & Wilkins Co , Baltimore, 1939 Ed by E Allen, p 740
(161) Tyslowitz, R Science 98 225, 1943
(162) Van Dyke, H B The physiology and pharmacology of the pituitary body Univ of Chicago Press, Chicago Vol I, 1936, Vol II, 1939
(163) Van Dyke, H B and Z Wallen-Lawrence J Pharmacol and Exper Therap 40 413, 1930
(164) Wallen-Lawrence, Z J Pharmacol and Exper Therap 51 263, 1934
(165) White, A Ann N Y Acad Sci 43 341, 1943
(166) White, A In The chemistry and physiology of hormones 1944 Science Press, Lancaster, pp 1–25
(167) White, A , R W Bonsnes and C N H Long J Biol Chem 143 447, 1942
(168) White, A , H R Catchpole and C N H Long Science 86 82, 1937
(169) White, A and L S Ciereszko J Biol Chem 140 cxxxix, 1941
(170) White, A and G I Lavin J. Biol Chem 132 717, 1940
(171) Wiley, F H and H B Lewis J Biol Chem 86 511, 1930
(172) Zondek, B and S Aschheim Arch Gynäkol 130 1, 1927

# ORGANIZATION OF THE RESPIRATORY CENTER

ROBERT F PITTS

*Department of Physiology, Cornell University College of Medicine New York N Y*

Quiet inspiration is an active process involving contraction of the diaphragm and the external intercostal muscles To accommodate the downwardly displaced viscera the tone of the abdominal muscles is reduced As air enters the respiratory passages the vocal cords abduct and the bronchioles dilate In contrast, quiet expiration is largely a passive process involving elastic recoil of the lungs, chest wall and diaphragm, assisted to some degree by the return of abdominal tone The volume of air moved at each respiration and the frequency of repetition of the movements are nicely adjusted to the metabolic demands of the body As these demands increase, more forcible contraction of the muscles of inspiration and active participation of both expiratory and accessory inspiratory muscles move the greater volumes of air necessary to meet the demands. The simultaneous contraction of many muscles deriving their motor excitation from cranial, cervical and thoracic segments, the alternation of activity of inspiratory and expiratory muscles, and the adjustment of the activity of these muscles to the needs of the moment necessitate some central co-ordinating and integrating neural mechanism This mechanism is commonly called the respiratory center The purpose of this review is to summarize the available information concerning the morphology of the respiratory center and the properties of its constituent neurons, to develop an adequate concept of its functional organization and to point out some of the obvious avenues of experimental approach which might lead to a sounder understanding of the central mechanisms of breathing

MORPHOLOGY OF THE MEDULLARY RESPIRATORY CENTER. The neural mechanisms responsible for the maintenance of rhythmic breathing understandably intrigued the early naturalists.[1] Galen (2) observed that section of the spinal cord in its upper reaches instantly abolished respiration a fact confirmed by Lorry in 1760 (3) But Legallois in 1812 (4) was the first to attempt a more precise localization of the respiratory center By removing parts of the brain of young rabbits he observed that breathing depends not upon the brain as a whole, but upon a circumscribed region of the medulla oblongata near the origin of the pneumogastric nerves Indeed Legallois found that the cerebellum and a part of the upper bulb could be removed without seriously affecting breathing

The last half of the 19th century was a period of intense interest and of major advance in knowledge of the morphology of the respiratory center During this period the anatomical limits assigned to the center were progressively expanded Thus Flourens (5) (6) described a vital node, the destruction of which abolished respiratory movements in both the trunk and face The vital node presumably approximated a mathematical point for it could be destroyed by thrusting a

[1] For an interesting and complete review of the early work on the respiratory center the paper of Cordier and Heymans (1) should be consulted

punch less than a millimeter in diameter into the apex of the calamus scriptorius Later he (7) redescribed the node as a bilateral structure, extending some 2 5 mm on either side of the midline in the caudal part of the medulla Schiff (8) (9) maintained that Flourens' vital node could be extirpated with impunity but that the ablation of the superior and external parts of the ala cinerea arrested respiration on the corresponding side of the body Gierke (10) contested the localization of Schiff, maintaining instead that deeper structures, namely, the nuclei and the fasciculi solitarii (or respiratorii, as he termed them), constituted the essential controlling mechanisms Mislawsky (11) believed that the central nucleus of the medullary raphe represented the respiratory center

In contrast to the above mentioned views identifying the respiratory center with discrete medullary structures, there developed the concept that the center is diffuse and represented by the scattered neurons which populate the reticular formation of the caudal half of the bulb Longet (12) was able to destroy the pyramids and restiform bodies without affecting respiration, whereas destruction of the reticular formation at the same level arrested breathing instantly Utilizing methods involving serial transections, localized lesions and gross stimulation, Bechterew (13), Kronecker and Marckwald (14), Marckwald (15), Gad and Marinesco (16) (17), Kohnstamm (18), Arnheim (19) and others had by the end of the last century contributed a mass of confirmatory evidence which firmly established the respiratory function of the reticular formation More recently this view has been confirmed, considerably strengthened, and extended by the application of exacting physiological techniques not available to the earlier investigators Thus Gesell, Bricker and Magee (20) (21) recorded, by means of fine needle electrodes thrust into the interior of the brain of a dog, the spontaneous nerve action potentials associated with breathing Monnier (22), Pitts, Magoun and Ranson (23), Magoun and Beaton (24), Pitts (25), Beaton and Magoun (26) and Brookhart (27) using similar needle electrodes stimulated the interior of the brain of the cat, monkey and dog with repetitive electric shocks, and recorded the respiratory responses which were obtained Comroe (28) injected into the brain of the cat minute quantities of buffered bicarbonate solutions of high carbon dioxide pressure and noted the respiratory responses induced In each instance the sites at which potentials were recorded, the sites of stimulation, or the sites of injection were identified in serial sections of the brain, and maps were made defining the precise limits of the respiratory center Such a map of the respiratory center of the cat is shown in figure 1 Although the limits of the center vary somewhat in the cat, monkey and dog, as might be expected from gross differences in the morphology of the bulb, its general confines are remarkably similar in the several forms Generally speaking, the respiratory center encompasses the medial and lateral reticular fields overlying the rostral half to two-thirds of the inferior olivary nuclei In terms of more easily identifiable external landmarks, the center underlies the caudal third of the floor of the fourth ventricle from the posterior borders of the auditory tubercles rostrally, to the obex or slightly below caudally The center is bilateral and extends from the midline to the restiform bodies It is limited to the reticular substance deep to the nuclear masses which

form the floor of the fourth ventricle In the cat it has a volume of some 30 to 50 cu mm. According to Finley (29) who studied the neuropathology of two cases of central respiratory failure, the location of the respiratory center in man is approximately the same

It is generally accepted that this medullary respiratory center with its afferent and efferent connections constitutes the minimum neural complement necessary for co-ordinate respiration and basic respiratory adjustments. Thus an animal whose brainstem has been transected just rostral to the respiratory center, exhibits hyperpnea when subjected to hypercapnia, anoxia and noxious stimulation, and apnea when hyperventilated or when the lungs are forcibly distended (30) (31) (32) (33) That more rostral levels of the brain including the pons (34) (35) (36) (37), midbrain (15) (38) (39), diencephalon (40) (41) and cerebral cortex (42) (43) (44) exert a powerful modifying control over breathing is likewise

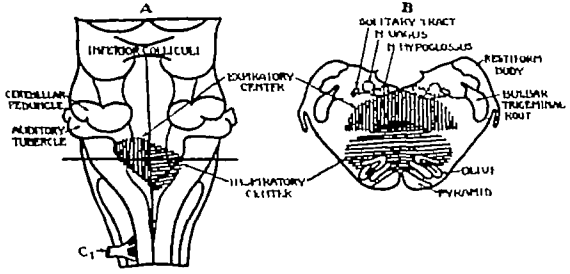



Fig 1 The location of the respiratory center in the cat A dorsal view of the lower brainstem after removal of the cerebellum B, transverse section of the medulla oblongata at the level indicated by the heavy horizontal line in A. (From Pitts R. F in Howell's Textbook of physiology (74), modified from Pitts Magoun and Ranson (23) )

generally admitted Whether one wishes to term these subsidiary respiratory centers, parts of a single but very diffuse respiratory center, or merely recognizes them as nervous mechanisms which play on and modify the activities of the medullary respiratory center is largely a matter of personal opinion. The reviewer prefers the latter concept There are, however, sporadic assertions, formulated usually on inconclusive evidence, that the basic control of breathing is mediated through suprabulbar levels of the brain (45) (46) (47), not by the medullary respiratory center

*Differentiation of inspiratory and expiratory divisions of the respiratory center* A majority of investigators have found evidence of a morphological differentiation of the respiratory center into inspiratory and expiratory divisions Thus Mislawsky (11), Kronecker and Marckwald (14), Gad and Marinesco (16), and Lewandowsky (48), utilizing gross methods of stimulation, were among the first to demonstrate the existence of separate inspiratory and expiratory centers. Recent workers, employing more precise experimental methods, have shown that

the expiratory division of the respiratory center occupies the dorsal reticular substance, the inspiratory division the ventral reticular substance Thus Nicholson (49) irrigated the floor of the fourth ventricle of the dog with cold Ringer's solution and found that expiration was immediately depressed, whereas inspiration was at least initially unaffected Irrigation with a dilute solution of cocaine (50) depressed expiration in a similar fashion, whereas stimulants such as nicotine and lobeline excited expiratory apnea As the depressing agents (cold and cocaine) acted for longer periods of time and penetrated to greater depths, inspiration was affected as well Polarizing currents (51) applied to the fourth ventricle produced respiratory effects which also indicated the existence of a superficial expiratory center and a deeper inspiratory center

Localized electrical stimulation has similarly provided evidence in the cat and in the monkey of morphologically distinct subdivisions of the respiratory center Pitts, Magoun and Ranson (23) using repetitive shocks of moderate intensity and high frequency[2] observed that excitation of the dorsal reticular formation in the cat produced tonic expiration, maintained for the duration of stimulation When the electrodes were advanced by as little as 0 5 mm into the ventral reticular formation, the response changed to one of maximal tonic inspiration, likewise maintained for the duration of stimulation Using these two responses to differentiate inspiratory and expiratory centers, the following relationships, diagrammatically illustrated in figure 1B, were determined The expiratory center lies in the dorsal reticular formation just beneath the subventricular gray matter, dorsal to, extending slightly cephalic to, and cupped over the cephalic end of the more ventrally placed inspiratory center Beaton and Magoun (26) have shown that the centers are similarly differentiated in the monkey, although there are minor differences in morphology in the two forms In the monkey the inspiratory center is confined to the ventro-medial reticular substance, and the expiratory center surrounds it dorsally, laterally, rostrally and caudally

In contrast, Brookhart (27), Gesell (52) and Bernthal (53) maintain that the neurons which constitute the inspiratory and expiratory centers are intimately intermingled and give no evidence of morphological segregation This concept is based upon their observations 1, that electrical potentials tapped from the lower brainstem of the dog through fine needle electrodes are not spacially grouped into those synchronous with inspiration and those synchronous with expiration, but appear to have a random distribution (20) (21), 2, that when the brainstem of the dog is stimulated with low frequency shocks of just threshold intensity,[3] no characteristic spacial distribution of inspiratory and expiratory responses is observed (27) These investigators claim that the differentiation

[2] Stimuli were condenser discharges of an intensity of 8 volts, a frequency of 240 per second and a time constant of 0 1 m sec The frequency employed is near the optimum for shocks of such wave form

[3] Shocks of an intensity of 0 5 to 1 5 volts and a frequency of 90 per second were employed Since intensity approached threshold and frequency was well below the optimum, it is not surprising that relatively minor changes in rate and amplitude of respiration were observed

described above, based on exploratory stimulation with shocks of moderate intensity, can have no real meaning, for they infer a physical spread of stimulating current so great that any localization of bulbar structures is impossible Furthermore, they maintain that the maximal inspiratory and expiratory responses represent predominant excitation of neurons of one center, but because of their magnitude, such responses mask the effects of simultaneous excitation of adjacent neurons of the other center

Each of these criticisms has been refuted in subsequent experimental studies, and the differentiation of inspiratory and expiratory centers has been reaffirmed Thus physical spread of current about the stimulating electrodes, when shocks of an intensity of 8 volts are employed, occurs within a sphere of tissue only 0.5 to 0 7 mm in radius (24) (25) Structures of known morphology can be localized within the brainstem with an accuracy of $\pm 0$ 5 mm , presumably the inspiratory and expiratory centers can be defined with equal accuracy (25) Phrenic motor neurons are caused to discharge impulses at high frequencies when the inspiratory center is stimulated, and are inhibited when the expiratory center is stimulated Mixed responses such as those postulated above, i e , phrenic nerve discharge during expiratory center stimulation, or inhibition of discharge during inspiratory center stimulation, were never observed (25) (54) (55) Stimulation of the inspiratory center with shocks of just threshold intensity causes increased frequency of discharge of phrenic motor neurons, some increase in respiratory mid volume and some acceleration of respiration Stimulation of the expiratory center produces the reverse effects (54) If such minor responses are used as criteria for differentiating the inspiratory and expiratory centers, the localization obtained using stimuli of an intensity of 0 9 volt is the same as that described previously using stimuli of 8 volts (24) It would appear, therefore, that a re examination of the respiratory center in the dog using adequate stereotaxic methods and stimuli of moderate intensity and high frequency would be worthwhile Comparative physiological studies on lower vertebrate forms would likewise contribute to a broader understanding of the morphology of the respiratory center

One valid criticism that may be directed against the stimulation method as a means of localizing a motor center within the brainstem is that it does not necessarily distinguish between the true motor center, its emergent efferent pathways, and those afferent tracts which play upon it However, it has been shown in various ways that the maximal inspiratory and expiratory responses ascribed to stimulation of the respiratory center actually result from activation of the center and not from excitation of afferent or efferent connections Thus stimulation of known afferent and efferent pathways rostral or caudal to the respiratory center does not yield the maximal respiratory responses which are characteristic of the center (23) Furthermore a comparison of the responses obtained on stimulation of the two sides of the medulla some 2 weeks after intracranial section of the glossopharyngeal and vagal rootlets on one side indicates that the intrabulbar terminations of these cranial afferents are in no wise involved in the responses (24) A comparison of the respiratory responses obtained after hemisection of the spinal cord at the first cervical segment similarly indicates that the efferent

pathways are not involved (23) Finally the identity of the responses obtained on stimulating the two sides of the medulla after chronic hemisection of the pons demonstrates that motor pathways descending from higher levels are not implicated (24)

*Interrelations of the inspiratory and expiratory centers* The reticular formation of the caudal portion of the bulb consists of motor type neurons scattered through a complex meshwork of ascending, descending and crossing nerve fibers so intricately interlaced as to defy histological analysis In the cat the neurons of the ventral reticular substance form a diffuse aggregation termed the *inferior reticular nucleus*, which is co-extensive with the physiologically defined inspiratory center (56) The neurons of the dorsal reticular substance are smaller, fewer in number, and show no definite nuclear grouping Kappers (57) has pointed out that neurons tend to be grouped into definite nuclei only when they receive their excitation from some single afferent system Since the neurons of the respiratory center receive impulses from many ascending and descending tracts and from cranial nerves as well, it is not surprising that they are diffusely distributed throughout the reticular substance

Despite this diffuse distribution, the neurons are intimately united into functional units by virtue of their rich synaptic interconnections These linkages are of two types, excitatory and inhibitory The excitatory linkages connect the constituent neurons of a given center, co-ordinate their activities, and provide for the simultaneous contraction of widely distributed respiratory muscles on the two sides of the body In contrast the inhibitory linkages connect the two centers and provide for the inhibition of one group of respiratory muscles during the contraction of the group of opposite function

The functional significance of these linkages is emphasized by the observation that all of the inspiratory muscles of the body can be caused to contract maximally by the electrical excitation of only a small fraction of the inspiratory center, less than one-thirtieth of the whole (36) The simultaneous stimulation of a similar small fraction of the center on the opposite side of the midline causes no increase in the depth of inspiration Hence all of the neurons of the inspiratory center can be excited by impulses transmitted synaptically from a small stimulated focus Since inspiration is maintained without interruption by rhythmic respiratory movements for the duration of stimulation, it is obvious that all of the neurons of the expiratory center are simultaneously inhibited The inspiratory center is the dominant division of the respiratory center, for when the two centers are stimulated simultaneously the response obtained is inspiration (36)

The importance of the linkages between the two halves of the respiratory center in co-ordinating the respiratory activities of the two sides of the body was demonstrated a number of years ago Lewandowsky (48) and Langendorff (58) (59) showed that if a deep sagittal incision is made in the midline in the caudal part of the bulb to sever these connections, and if the pulmonary afferents are eliminated by section of the vagus nerves, independent respiratory rhythms develop on the two sides of the body Furthermore in such a preparation central

stimulation of one vagus nerve inhibits breathing only on one side of the body It is apparent that synchrony in contraction of the respiratory muscles on the two sides depends on local connections which link the two halves of the respiratory center at the bulbar level

In normal eupneic respiration, only the inspiratory phase is active, but when breathing is stimulated, both inspiratory and expiratory muscles participate Under such conditions the two groups of muscles contract alternately, and this alternation of contraction is an obvious consequence of inhibitory connection which links the two centers The orderly and synchronous contraction of muscles of the facial, glossopharyngeal, laryngeal, pectoral, thoracic and abdominal groups on the two sides of the body is on the other hand a consequence of the excitatory connections which link the constituent neurons of each center

*Descending respiratory pathways* The neurons of the respiratory center send their axons to the nuclei of the 5th, 7th, 9th, 10th, 11th and 12th cranial nerves which supply the striated muscles of the jaws, mouth, nares, pharynx, larynx and tongue and the smooth muscles of the bronchial tree Their axons likewise descend through the cervical and thoracic spinal cord to supply the diaphragm and the intercostal and abdominal muscles These axons, forming the descending respiratory motor tracts, course through the anterior and anterolateral columns of the spinal cord and for the most part are uncrossed (23) (56) (60) (61) Section of these pathways on one side at $C_1$ causes ipsilateral respiratory paralysis, section on both sides causes immediate complete respiratory failure and death *In man traumatic lesions of the anterior columns of the spinal cord, as a consequence of fracture of the cervical spine, may cause such a high degree of respiratory inadequacy and anoxia as to lead to the development of a progressive confusional syndrome (62)*

Although the initial ipsilateral respiratory paralysis from hemisection of the cord at $C_1$ is complete, recovery of function on the side of the lesion is prompt, and after 5 to 6 weeks both halves of the thorax and both leaves of the diaphragm become equally active (56) Obviously crossed connections exist within the spinal cord between the respiratory neurons on one side of the bulb and the effector motor nuclei on the opposite side of the spinal cord Why some 4 to 6 weeks are necessary normally for establishment of their function is something of a mystery According to Porter (63) that leaf of the diaphragm which is paralyzed by hemisection of the cord becomes immediately active when the opposite phrenic nerve is cut and the opposite leaf paralyzed Deason and Robb (64) claim that crossing of impulses in the cord at a spinal level under these conditions is a function of the intensity of discharge by the inspiratory center Thus in hyperpneic respiration crossing occurs without section of the opposite phrenic, and with section of the phrenic, crossing apparently results from the dyspneic condition of the animal In contrast, Rosenblueth et al (65) believe that mere blockage of phrenic motor impulses, not central respiratory stimulation or even loss of afferent impulses from the diaphragm, produces a local spinal change favoring crossing Some very fundamental yet unrecognized characteristic of central nervous integration must underlie the crossed phrenic phenomenon of

Porter Elucidation of this phenomenon would be a valuable contribution to neurophysiology

THE NATURE OF THE RESPIRATORY NEURAL DISCHARGE The neural mechanisms by which the inspiratory and expiratory centers control the contractions of respiratory muscles have been elucidated by studies of impulses carried by individual motor fibers of peripheral respiratory nerves Adrian and Bronk (66) and Bronk and Ferguson (67) dissected small twigs composed of a few fibers from the phrenic and intercostal nerves, and recorded from them spontaneous potentials associated with breathing It was found that trains of impulses of low frequency, from 5 to 30 per second in eupnea, descend over the motor fibers to innervate the respiratory muscles Such frequencies of excitation are far below those necessary to produce fused tetanic contractions of the individual muscle fascicles However, the impulses carried by the several motor fibers are out of step and their muscle units twitch asynchronously Hence the respiratory contraction as a whole is smooth and fluid In small animals such as mice and rats the average frequency of impulses in eupneic breathing is much higher than in larger animals such as the dog and horse (68) The total number of impulses discharged by an average neuron in a single respiratory cycle is about the same in all forms, for the cycle is much shorter in small animals than in large ones

The finding of asynchronous discharge of impulses at low frequencies when recordings are made from small twigs containing few fibers stands in sharp contrast to the inferences drawn from recordings made from whole nerve trunks Rijlant (45) (47) 69), studying potentials in the phrenic trunk, maintains that there is a high degree of synchronization of the individual motor neurons and a basic rhythm of discharge of about 90 per second. While the lack of synchrony evident in the potentials of few fiber preparations does not negate some degree of synchrony in whole nerves, certainly discharge frequencies of 90 per second in individual fibers are observed only in conditions of extreme dyspnea

*Motor mechanisms grading depth of breathing* Three changes in the character of the respiratory motor discharge are responsible for the increase in amplitude and force of contraction of the respiratory muscles in hyperpnea The individual motor neurons discharge impulses at higher frequencies, previously quiescent neurons are recruited, and the duration of the trains of impulses discharged by the individual neurons is increased (33) (54) (66) (67) (70) (71) An increase in impulse frequency from 20 to as high as 100 per second or more has been observed in phrenic motor neurons following clamping of the trachea (66) But probably of more significance in increasing respiratory depth is the factor of recruitment, for in eupneic breathing only a small fraction of the inspiratory motor neurons and even fewer expiratory motor neurons discharge impulses With respiratory stimulation these quiescent neurons are progressively recruited, and the total force of respiratory contraction increases accordingly In eupnea many inspiratory neurons discharge only a few impulses at the peak of inspiration, but in hyperpnea these neurons begin to discharge early in the inspiratory cycle Thus additional muscular force applied at the start of inspiration speeds the intake of air

The statement of Adrian and Bronk (66) that recruitment of phrenic motor neurons plays no rôle in the increased diaphragmatic excursion of hyperpnea is frequently referred to, but has no basis in fact, for recruitment has been repeatedly observed in subsequent studies (33) (54) (68) (70) Their error is perhaps a tribute to their technical skill in dissecting from the phrenic trunk what truly must have been single nerve fibers The reviewer in attempting similar dissections has usually split off twigs composed of several fibers, which invariably demonstrated recruitment

*The pattern of respiratory motor discharge* Gesell et al (20) (21) (68) (70) (72) have pointed out that the frequency of impulses discharged by inspiratory neurons characteristically is low at the start of inspiration, and gradually augments as the cycle proceeds, to reach a maximum at the peak of inspiration Some neurons discharge throughout the inspiratory cycle, others are recruited only after inspiration gets well under way Such a pattern of impulses provides for a muscular contraction of gradually increasing strength to overcome the increasing elastic resistance of the expanding thorax. The inspiratory discharge ceases abruptly at the end of inspiration, freeing the potential energy stored in the thorax in the form of elastic tension Air is expelled from the lungs, at first rapidly and then more gradually, as this store of potential energy is dissipated

When breathing is stimulated so that expiration becomes an active process, the pattern of impulses discharged by expiratory neurons may be seen to differ from that of inspiratory neurons The frequency characteristically is high at the start of expiration and gradually declines as the chest volume is reduced The application of maximum muscular force at the start of expiration speeds the expulsion of air early in the cycle when thoracic volume is greatest Some expiratory neurons, however, discharge at a constant rate throughout the expiratory cycle, starting and stopping with equal abruptness Gesell believes that these units may be more concerned with the maintenance of proper visceral and body posture than with the movement of air

RHYTHMIC PROPERTIES EXHIBITED BY THE RESPIRATORY CENTER. The neurons of the respiratory center exhibit two types of rhythmic processes, the rhythmic discharge of trains of repetitive impulses and the rhythmic alternation of phases of activity and quiescence The former we shall term the *rhythm of the generation of impulses*, the latter, the *rhythm of breathing*

*The rhythm of the generation of impulses* The neurons of the respiratory center are sensitive to their chemical environment, showing a type of specialization not dissimilar to that of certain peripheral chemical receptor elements such as the taste buds or the carotid glomus receptors Specifically these neurons are sensitive to the carbon dioxide pressure of the arterial blood, responding with an increased rate of discharge to a rise in arterial partial pressure They are probably likewise sensitive to changes in the hydrogen ion concentration, temperature, oxygen pressure and rate of flow of the arterial blood which perfuses the medulla But like other neurons they are excited by nerve impulses delivered to them over collaterals of the major ascending afferent tracts, the entering sensory divisions of the cranial nerves and a number of descending motor projection systems

The rate of discharge of impulses and the numbers of neurons active is finally determined by the sum of all the excitatory and inhibitory influences acting on them For any given short interval of time, the sum of these influences can be considered as more or less constant How does such a constant stimulus acting on the respiratory neurons cause them to discharge repetitive impulses, and how does an increase in such a stimulus increase their rates of discharge?

Adrian and Zotterman (73) proposed a scheme to explain the repetitive discharge of peripheral receptor organs when acted upon by a constant stimulus, which with appropriate modifications has been applied by Pitts (74) to the respiratory center Figure 2 presents in diagrammatic form the basic elements of this scheme When a motor neuron discharges an impulse it becomes absolutely refractory for a period of 1 or 2 milliseconds, and then gradually recovers its excitability during a relatively refractory period lasting many milliseconds Eventually its excitability returns to normal (75) A hypothetical curve of

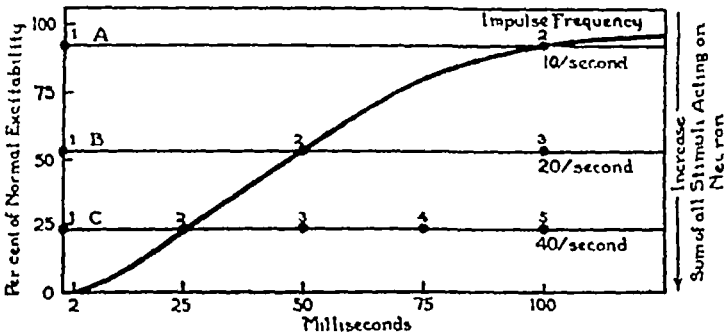



Fig 2 A diagrammatic representation of the mechanism of repetitive activity of a respiratory motor neuron based upon the time course of its recovery of excitability following the discharge of an impulse (From Pitts, R F , in Howell's Textbook of physiology (74) )

excitability for a single neuron of the respiratory center following the discharge of an impulse is plotted in figure 2 in bold face An absolutely refractory period of 2 milliseconds during which excitability is zero, is followed by a relatively refractory period of 100 or so milliseconds, during which excitability returns gradually to its normal resting value We may plot as horizontal lines A, B and C, three levels of maintained excitation, representing the sum of all stimuli acting upon the neuron under three different conditions Let us assume that such stimuli acting on the neuron do not prevent it from recovering its excitability, and let us further assume that a second impulse will be discharged when the excitability of the neuron recovers sufficiently so that its threshold is exceeded by the sum of all the stimuli acting upon it It is obvious from figure 2 that a second impulse will be discharged after 100 milliseconds at intensity A, after 50 milliseconds at intensity B, and after 25 milliseconds at intensity C Following the discharge of this second impulse the entire cycle is repeated It is thus evident that impulses will be discharged repetitively, and that an increase in the intensity of the stimulus acting on the neuron will cause it to discharge at a higher frequency

For the intensities indicated on the graph, the hypothetical discharge frequency will vary from 10 to 40 impulses per second

Gesell (52) (76) has expanded this view and developed an electrotonic concept of the mode of origin of rhythmic impulses There is assumed to exist a potential gradient between the dentritic processes and the axon hillock of the neuron such that current flows between these points[4] As shown in figure 3 the current flows from dendrites to axon hillock within the soma and in the reverse direction in the surrounding medium This electrotonic current emerging from the axon hillock is presumed to constitute the maintained stimulus referred to above, and to cause the repetitive generation of impulses at this point The magnitude of this current, which fundamentally determines the frequency of

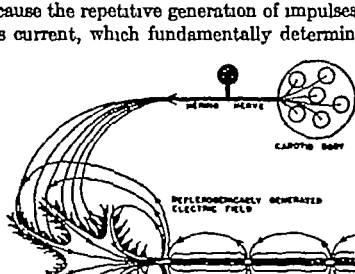



Fig 3 A diagrammatic representation of the electrotonic concept of nerve cell discharge (From Gesell, R., Science (76) )

discharge of the neuron, is affected both by the chemical environment of the cell and by the nerve impulses impinging on the cell.

The mode of action of this chemical environment, specifically that of carbon dioxide, has received considerable attention Haldane (77) believed that the respiratory center is exquisitely sensitive to the hydrogen ion concentration of the blood and that carbon dioxide acts as a stimulant by virtue of its properties as an acid when dissolved in blood, a view recently revived by Banus et al (78) Gesell (79) maintains that respiration is regulated not by the hydrogen ion concentration of the blood but by that of the interior of the respiratory neurons into which carbon dioxide, but not other acids, diffuses rapidly Nielsen (80) and Schmidt and Comroe (81) hold that carbon dioxide as a molecular species acts as a respiratory stimulant independent of its properties as an acid Recently it has been shown that carbon dioxide is metabolized by certain tissues (82) altering their rates of oxygen consumption (83) and rates of glycolysis (84) Perhaps such metabolic effects of carbon dioxide underlie its stimulating action

[4] This concept of longitudinal polarization of the nerve cell body was first clearly expressed by Gerard (139) (140)

Within the past few years Gesell and his co-workers have developed an acid-humero-electrotonic theory capable of explaining on a unitary basis both chemical and nervous excitation of respiratory neurons Acetylcholine deposited at the synapses by impinging nerve impulses is presumed to generate the potential differences upon which the flow of electrotonic current depends They postulate that the potential difference at any moment depends upon the total quantity of acetylcholine *pooled at the neuron* *This total humoral pool is the resultant* of the rate of its synaptic liberation, i e , number of impinging impulses, and its rate of destruction, i e , the activity of cholinesterase An increase in the hydrogen ion concentration of the blood and body fluids diminishes the rate of enzymatic breakdown of acetylcholine, resulting in a greater pooling of the humor at the respiratory neurons Thus the stimulating effects on breathing of an increase in hydrogen ion concentration of the blood whether produced by acid or by the inhalation of carbon dioxide is envisioned to be the consequence of its anticholinesterase activity (85, 86)

One rather intriguing aspect of this theory lies in its explanation of both excitation and inhibition on the basis of a single neurohumor [5] Thus excitatory effects are mediated through those synapses which deposit acetylcholine on the dendrites and soma of the neuron, increasing the potential difference between these regions and the axon hillock An increase in potential difference increases the flow of electrotonic current and the rate of discharge of impulses On the other hand inhibitory effects are mediated through those synapses which impinge on the axon hillock The deposition of acetylcholine in this region diminishes the potential difference between dendrites and hillock, thus reducing the flow of electrotonic current and the rate of generation of impulses

The theory of acid-humero-electrotonic control of breathing has been tested by rather indirect means A number of simple model systems such as the cold-blooded heart (87), rectus abdominis muscle (88), and salivary gland (87) behave with respect to nerve stimulation, application of acetylcholine and acidification in accordance with the theory Similarly the respiratory effects of peripheral nerve excitation, inhalation of carbon dioxide and administration of eserine and atropine may be broadly interpreted as supporting the hypothesis (90) (91) However, the theory if correct can be firmly established only by devising critical experiments which will test directly in the respiratory center each of its basic tenets This will be a technical problem of no mean proportions

*The rhythm of breathing* There have developed two divergent views of the factors underlying the rhythm of breathing On the one hand this rhythm is considered to be an expression of an inherent automaticity of the neurons of the respiratory center, i e , the individual respiratory neurons when freed of all afferent regulatory control, still discharge impulses in rhythmically repeated trains On the other hand this rhythm is considered to be an expression of the periodic interruption of a maintained tonic discharge of the medullary respiratory

[5] The basic concept of excitatory and inhibitory poles of the neuron had been developed previously by Gerard (139) (140) and is equally applicable whether the synaptic exciter is humoral or electrical

center by two inhibitory mechanisms, the one a vagal reflex mechanism, the other a brain stem mechanism, the *pneumotaxic center*

The concept of inherent rhythmicity of the respiratory center is based upon the following lines of evidence Rosenthal (92) found that breathing, although modified, remains rhythmic after transection of the brainstem between the superior and inferior colliculi and section of the spinal dorsal roots and cranial nerves Winterstein (03), Adrian and Bronk (66) and Gesell (79) noted that rhythmic bursts of impulses can be recorded from the respiratory motor nerves of curarized animals in which all respiratory movements had been abolished Adrian and Buytendijk (94) observed rhythmic potentials of a frequency approximating that of the normal respiratory movements in the isolated brainstem of the goldfish Heymans and Heymans (95) noted that rhythmic respiratory movements of the larynx persist in the isolated perfused head of the dog Since these procedures, involving more or less complete surgical and pharmacological deafferentation of the brainstem, do not abolish the rhythm of breathing, the conclusion that the respiratory center is inherently rhythmic seems superficially to be a reasonable one It must be realized, however, that the evidence is of a negative sort, i.e , these stated procedures do not abolish the rhythm of breathing In fact, as will be evident subsequently, none of them abolish the inhibitory functions of the pneumotaxic center

A number of theories have been advanced to account for this presumed automaticity of respiratory neurons Thus Adrian and Buytendijk postulated that the respiratory neurons are progressively depolarized during activity and repolarized during quiescence Head (96) implied that the neurons contain some limited store of energy-producing materials which are utilized during the phase of activity and restored during quiescence Gesell (52) believed that summation of subnormality during the phase of activity reduces excitability to a point where discharge ceases, whereas normal excitability is recovered during quiescence.

Evidence that the rhythm of breathing is impressed upon the neurons of the respiratory center by inhibitory mechanisms acting from without was first advanced by Marckwald (14) (15) (97) He observed that if the brainstem of a rabbit is transected through the pons, i e , below the inferior colliculi, respiration remains normal so long as the vagus nerves are intact But upon section of the vagi, the animal inspires deeply and the chest is held in a fixed position of inspiration until death ensues from asphyxia If the transection is made at a higher level, i e , between the superior and inferior colliculi, section of the vagus nerves merely leads to slowing and deepening of breathing Therefore there lies between these two levels of transection, presumably in the inferior colliculi, a mechanism capable of maintaining rhythmic breathing when the vagus nerves are cut Marckwald concluded that the medullary respiratory center has no inherent rhythm of its own but mediates maintained tonic inspiration, or inspiratory cramp as he termed it Two inhibitory mechanisms, the one a vagal reflex mechanism, the other a mechanism located in the inferior colliculi, serve to interrupt this inspiratory cramp and convert it into rhythmic respiration Either mechanism alone is capable of conferring rhythmicity on the respiratory center In normal breathing the vagal inhibitory mechanism plays the major rôle

The findings of Marckwald were confirmed by a number of his contemporaries including Loewy (98), Asher and Luscher (99), Lewandowsky (48) and others, But Schrader (100) and Langendorff (101) studying respiration in the frog failed to obtain evidence of either inhibitory mechanism In general the results of Lumsden (102, 103, 104) on the cat are in accord with the views of Marckwald with the following noteworthy exceptions Lumsden placed the brainstem inhibitory mechanism in the rostral few millimeters of the pons, not in the inferior colliculi as had his predecessors Furthermore he maintained that the exclusion of this region, which he termed the pneumotaxic center, abolished rhythmic breathing even though the vagi were intact The inspiratory cramps or apneuses as he called them represent the release of the inspiratory center from rhythmic inhibition by the pneumotaxic center

Observations of Henderson and Sweet (105), Teregulow (106) and Hess (107) were more in accord with those of Marckwald than with those of Lumdsden However they interpreted apneuses as postural manifestations of decerebrate rigidity, not as respiratory phenomena In somewhat the same vein Nicholson and Hong (108) have recently described, as a consequence of pontile decerebration and vagal section, an increase in inspiratory tone of the thorax with superimposed rhythmic respiratory movements

The earlier literature on the rôle of the pneumotaxic and vagal reflex mechanisms in contributing rhythmicity to breathing is both confusing and intriguing That the vagus nerves and a nervous mechanism located in the brainstem rostral to the medullary respiratory center together exert a profound influence on the rhythm of respiration is evident But the significance of these mechanisms has long been a subject of controversy Recent investigations of Stella (30, 31, 35) and Pitts et al (32) (33) (36) have advanced our knowledge of these mechanisms and have permitted a formulation of their rôle in respiration In general the concepts originally outlined by Marckwald have been confirmed

It has been shown that the pneumotaxic center is a bilateral structure situated for the most part in the upper few millimeters of the pons (35) (36), but extending in reduced form as a scattered collection of neurons down into the medulla (33) The persistence of this small medullary remnant in an animal whose brainstem has been transected through the pons may account for the very slow apneustic rhythm of breathing which has been observed occasionally after vagal section The pneumotaxic center is connected with the medullary respiratory center by ascending and descending pathways which course through the lateral parts of the medulla and pons ventral to the bulbar trigeminal tracts (36) Localized electrolytic lesions so placed as to interrupt these pathways, or transection of the brainstem caudal to the pneumotaxic center have no effect on eupneic breathing nor do these procedures alter the response of the medullary respiratory center to various repiratory stimuli (31) (33) (35) (36) Thus rate and depth of respiration increase in a normal fashion when carbon dioxide is administered (30), when cutaneous afferent nerves are stimulated (36), and when the carotid glomus is excited (31) The application of cold thermodes to block the vagus nerves or secion of these nerves abolishes rhythmic breathing and leads to the development of apneusis The depth of apneusis is increased by those same stimuli which

increase the rate and depth of rhythmic respiration Apneusis is, therefore, a respiratory phenomenon, and not a postural manifestation of decerebrate rigidity This view is borne out by the observation that transection of the brain stem immediately caudal to the inferior colliculi produces decerebrate rigidity, but since the pneumotaxic center remains intact, apneusis does not result when the vagi are subsequently blocked (32, 35)

One vagus nerve or one-half or less of the bilateral pneumotaxic center in functional connection with the medullary respiratory center is sufficient to maintain rhythmic breathing (32) When these inhibitory influences are completely abolished and apneusis produced, intermittent stimulation of the expiratory center or of the central end of one vagus nerve restores the rhythm of breathing (36) Both procedures effect a rhythmic inhibition of the tonically active inspiratory center, and thus convert the maintained inspiration into a series of interrupted inspiratory movements The pattern of the motor neural discharge responsible for these artificially induced respiratory movements is identical with that of normal rhythmic breathing (33) This fact provides strong support for the view that normal rhythmic breathing results from intermittent inhibition of the tonically active inspiratory center by vagal afferent impulses

FUNCTIONAL INTERRELATIONSHIPS OF THE RESPIRATORY COMPLEX The functional interrelationships of the medullary respiratory center, the vagal inhibitory mechanism and the pneumotaxic center have been summarized by Pitts (33) (74) The basic elements of this organization are presented in diagrammatic form in figure 4. The neurons of the respiratory center, sensitive to their chemical environment and to nerve impulses impinging upon them discharge impulses repetitively These impulses, transmitted over the central respiratory pathways to cranial and spinal motor nuclei, are relayed out over peripheral motor nerves to cause contraction of the respiratory muscles

The neurons of the inspiratory center are more excitable than are those of the expiratory center, and in eupnea breathing is largely an inspiratory process When respiration is stimulated either chemically or as a result of an increased inflow of excitatory afferent impulses, less excitable neurons are recruited and those previously active discharge at higher frequencies. Inspiration is deeper and fuller and expiration becomes active

When the medullary respiratory center is isolated from the major inhibitory influences mediated through the pneumotaxic center and the vagus nerves (cf dashed lines in fig 4 indicating transection of the pons and section of the vagus nerves) maintain inspiration or apneusis results. The more excitable neurons of the inspiratory center discharge a continuous series of impulses. Because of reciprocal connections between the two centers, the expiratory center is inhibited, and the rhythm of breathing is abolished The greater the chemical or synaptic drive acting on the respiratory center, the higher is the frequency of discharge of the individual inspiratory neurons, the greater the number of neurons active, and the deeper the maintained inspiration Thus regulation of the depth of breathing is dependent upon the properties and organization of the respiratory center and its peripheral effector pathways

As the lungs expand in inspiration the stretch receptors of the pulmonary

parenchyma are stimulated (109, 110) Impulses conducted centrally over afferent fibers of the vagus nerves (cf fig 3) excite the expiratory center (36, 111, 112) In eupnea the major effect of excitation of the expiratory center is inhibition of the inspiratory center, for expiration is largely passive As the lungs deflate the inflow of afferent impulses over the vagi subsides, and the inspiratory center released from inhibition again begins to discharge The respiratory cycle is then repeated

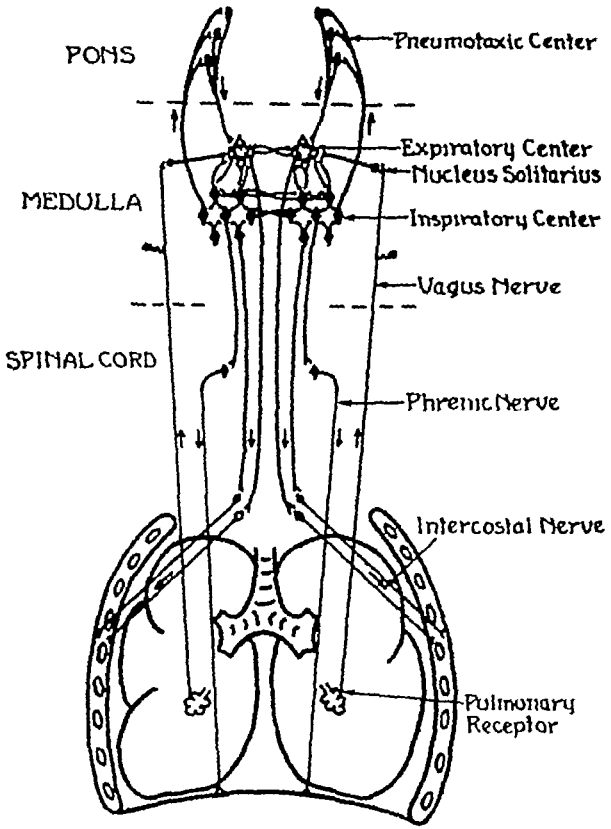



Fig 4 A diagrammatic representation of the organization of the basic neural mechanisms responsible for rhythmic respiration (From Pitts, R F , in Howell's Textbook of physiology (74) )

When breathing is stimulated, the more intense discharge of the inspiratory center causes a more rapid inflation of the lungs Because a greater barrage of afferent impulses is required to inhibit the inspiratory center, inspiration proceeds to a greater depth (109) Active expiration speeds the collapse of the lungs, and because the lungs are deflated to a greater degree receptors are stimulated which excite the succeeding inspiration (109, 110) The increase in velocity of both inspiration and expiration shortens the respiratory cycle and increases the rate of respiration The addition of the vagal inhibitory mechanism to the medullary respiratory center imparts rhythmicity to breathing and provides the basis for variations in the rate of respiration

The pneumotaxic center serves an inhibitory function much like that of the vagal reflex mechanism but differs in that it lies wholly within the brainstem (cf fig 4) Impulses originating within the inspiratory center are transmitted rostrally over ascending pathways in the lateral part of the tegmentum of the brainstem (32) These impulses are relayed through the pneumotaxic center and returned over descending pathways to excite the expiratory center When this barrage of impulses reaches a sufficient intensity, the inspiratory center is inhibited and the circuit ceases to function As activity of the expiratory center declines, the inspiratory center escapes from inhibition and the cycle is repeated In eupneic breathing the vagal mechanism is dominant and the pneumotaxic center subsidiary, whereas in the polypnea of thermal or decorticate panting the reverse is true (32, 113, 114) In all instances after section of the vagus nerves rhythmicity of breathing depends upon the inhibitory functions of the pneumotaxic mechanism Failure to eliminate this mechanism in the surgical and pharmacological deafferentations of the respiratory center described earlier (66, 70 92, 93, 94, 95), accounts for the persistence of rhythmicity of breathing which was erroneously ascribed to inherent automaticity of the medullary respiratory center

THE REGULATION OF THE ACTIVITY OF THE RESPIRATORY CENTER. An account of the nature and rôle of the many neural and chemical factors which regulate ventilation is beyond the scope of this review A number of excellent summaries have appeared within recent years which the interested reader may consult for detailed information (1, 52, 53, 79, 81, 115 116, 117) Suffice it to indicate here in general terms how these several factors affect the neurons of the respiratory center

*Sensitivity of the respiratory neurons to their environment* That carbon dioxide is the chemical stimulant par excellence of the neurons of the respiratory center was amply demonstrated by Haldane and his co-workers (118, 119, 120) In eupnea ventilation is so regulated as to maintain the partial pressure of carbon dioxide in the arterial blood perfusing the respiratory center at a relatively constant value near 40 mm Hg Any increase in carbon dioxide pressure causes an increase in the respiratory motor discharge, any decrease diminishes the discharge That these respiratory effects are mediated in large part through a direct action of carbon dioxide on respiratory neurons has been demonstrated by their persistence in undiminished form following denervation of the carotid and aortic chemo-receptors (121 122, 123)

The neurons of the respiratory center are likewise stimulated directly though to a lesser degree by a rise in temperature, by a decrease in flow and by an increase in acidity of the arterial blood perfusing the medulla A decrease in oxygen pressure on the other hand primarily depresses the respiratory neurons (121, 124)

*The nervous regulation of breathing* Except in conditions of eupnea, nervous mechanisms, both reflex and voluntary, play a more significant rôle in the regulation of ventilation than does the sensitivity of the respiratory center to its environment (115, 117) Even in eupnea, as pointed out above, the Hering Breuer inhibitory reflexes are intimately concerned with rhythmicity of breathing

It is generally agreed that respiratory stimulation in anoxia depends upon reflex excitation of the inspiratory and expiratory centers by impulses from the carotid and aortic glomi (121, 125, 126) transmitted to the respiratory center over the glossopharyngeal and vagus nerves These chemoreceptors are likewise sensitive to increases in carbon dioxide pressure and hydrogen ion concentration of the blood, although it is probable that their sensitivity is of a lower order of magnitude than is that of the neurons of the respiratory center (122, 123) Some claim that chemo-receptor reflexes play a more or less prominent rôle in the regulation of ventilation in eupnea (127, 128), others maintain that they are active only in such emergency states as anoxia, severe hypercapnia and acidosis (129)

The major ascending sensory tracts and cranial sensory roots give off collaterals to the inspiratory and expiratory centers in their passage through the medulla Proprioceptive afferents from thoracic muscles exert a regulatory control over the depth and rhythm of breathing (130, 131) Noxious stimuli applied to the skin and deeper somatic structures excite hyperpnea, applied to the respiratory passages they produce apnea Passive movements of the limbs reflexly stimulate the respiratory center (132, 133) Distention of the great veins and right atrium by a rise in venous pressure increases ventilation (134) An increase in arterial pressure reflexly induces apnea, a fall leads to hyperpnea (135)

In man voluntary control of respiration is important in such activities as speaking, singing, whistling, blowing, straining, etc This control depends on regulatory nerve impulses impinging on the respiratory center over pathways which descend from the cerebral cortex (42, 43, 44) Of all the neural mechanisms which regulate the respiratory center, the voluntary mechanism is basically the most powerful *Thus by breathing as deeply and rapidly as possible* ventilation may be increased to as much as 150 liters per minute or more (136), a value in excess of that observed in severe muscular exercise to the point of exhaustion (137, 138)

The thermal regulatory center in the hypothalamus exerts a powerful control over the respiratory center in lower animals Thus the polypnea of thermal panting depends upon impulses which descend from the anterior hypothalamic areas to the pneumotaxic center, facilitating it and causing it to become the dominant factor in determining the respiratory rhythm (32, 113, 114) Emotional hyperventilation in man, which may in part be hypothalamic in origin and in part cortical, is often of an intensity sufficient to induce severe alkalosis and tetany

Ultimately the activity of the respiratory center is determined by the sum of all the excitatory and inhibitory factors playing upon it This is especially evident in exercise in which ventilation may increase to as much as 120 liters per minute Comroe (115) has pointed out that many types of stimuli must summate at the respiratory center to increase ventilation to such an extent Although specific circumstances may be cited in which one or another factor plays a dominant rôle in regulating respiration, the rate, depth and rhythm of breathing are finally determined by the summated contributions of all.

SUMMARY

The neurons which constitute the respiratory center are diffusely distributed through the reticular formation of the caudal half of the bulb Those in the ventral reticular substance overlying the inferior olivary nuclei are concerned with inspiration Those in the dorsal reticular substance are concerned with expiration Excitatory connections between the constituent neurons of a given center provide for co-ordination of contraction of widely distributed respiratory muscles Inhibitory connections between the two centers provide for alternation of contraction of inspiratory and expiratory muscles

The neurons of the respiratory center are sensitive to the chemical and physical constitution of their fluid environment They are excited by impulses impinging upon them by way of collaterals of the major sensory and motor tracts Under combined chemical and synaptic stimulation these neurons discharge impulses repetitively The more intense the chemical stimuli and the more numerous the synaptic stimuli, the greater is the frequency of discharge of impulses and the greater is the number of neurons active Since the inspiratory neurons have the lowest threshold, inspiration is the dominant phase of respiration In the absence of inhibitory influences which act upon the inspiratory center from without, its discharge is continuous, and maintained tonic inspiration or apneusis results The depth of the apneusis is a function of the sum of all the stimuli acting upon the neurons of the inspiratory center

Two inhibitory mechanisms operate to interrupt rhythmically this repetitive activity of the inspiratory center, namely, the vagal inhibitory mechanism and the pneumotaxic mechanism As the lungs inflate during inspiration, impulses which originate in pulmonary stretch receptors and which travel centrally over afferent fibers of the vagus nerves excite the expiratory center The inspiratory center is reciprocally inhibited and expiration results As the lungs deflate, the afferent inflow diminishes, the inspiratory center escapes and the cycle repeats The pneumotaxic mechanism plays an analogous although a subsidiary rôle under most circumstances Thus impulses originating in the inspiratory center are transmitted rostrally to the pneumotaxic center and are then relayed caudally to the expiratory center Excitation of the expiratory center leads to reciprocal inhibition of the inspiratory center, whereupon the circuit ceases to function and the cycle repeats. When the vagus nerves are cut the pneumotaxic center maintains the rhythm of breathing

The rhythm of breathing is impressed upon the respiratory center by inhibitory mechanisms operating from without, it is not an expression of properties inherent in the neurons of the center The depth of breathing is determined by the sensitivity of these neurons to their environment and by the excitatory influences exerted by the many afferents which impinge upon them The rate of breathing is determined by the excitability of the neurons of the center and by the activity of the inhibitory mechanisms which rhythmically interrupt their repetitive discharge

(103) LUMSDEN, T J Physiol 57 354, 1923
(104) LUMSDEN, T J Physiol 58 81, 1923
(105) HENDERSON, V E AND T A SWEET Am J Physiol 91 94, 1929
(106) TEREGULON, A G Pflüger's Arch 221 486, 1929
(107) HESS, W R Die Regulierung der Atmung gleichzeitig ein Betrag zur Physiologie des vegitativen Nervensystems Leipzig, G Thieme, 1931
(108) NICHOLSON, H C AND J HONG Fed Proc 1 63, 1942
(109) ADRIAN, E D J Physiol 79 332, 1933
(110) KNOWLTON, G C AND M G LARRABEE Am J Physiol 133 351, 1941
(111) LARRABEE, M G AND G C KNOWLTON Am J Physiol 133 360, 1941
(112) GESELL, R AND C MOYER Am J Physiol 131 674, 1941
(113) KELLER, A D Am J Physiol 89 289, 1929
(114) KELLER, A D J Neurophysiol 1 543, 1938
(115) COMROE, J H Physiol Rev 24 319, 1944
(116) GESELL, R Ann Rev Physiol 1 185, 1939
(117) SCHMIDT, C F AND J H COMROE Physiol Rev 20 115, 1940
(118) HALDANE, J S AND J G PRIESTLEY J Physiol 32 225, 1905
(119) CAMPBELL, J M H, C G DOUGLAS, J S HALDANE AND F G HOBSON J Physiol 46 301, 1913
(120) DOUGLAS, C G AND J S HALDANE J Physiol 38 401, 1909
(121) GEMMILL, C L AND D L REEVES Am J Physiol 105 487, 1933
(122) COMROE, J H AND C F SCHMIDT Am J Physiol 121 75, 1938
(123) SCHMIDT, C F, J H COMROE AND R D DRIPPS Proc Soc Exper Biol 42 31, 1939
(124) SMYTH, D H J Physiol 88 425, 1937
(125) COMROE, J H Am J Physiol 127 176, 1939
(126) HEYMANS, C, J J BOUCKAERT AND L DAUTREBANDE Arch int Pharmacodyn 39 400, 1930
(127) HEYMANS, C AND J J BOUCKAERT Ergebn Physiol 41 28, 1939
(128) VON EULER, U S, G LILJESTRAND AND Y ZOTTERMAN Skand Arch Physiol 83 132, 1940
(129) SCHMIDT, C F In Macleod's Physiology in modern medicine, 9th ed St Louis, C V Mosby Co 1941
(130) COOMBS, H D AND F H PIKE Am J Physiol 45 569, 1918
(131) SHARPEY-SCHAFER, E J Physiol 75. 130, 1932
(132) HARRISON, W G, JR, J A. CALHOUN AND T R HARRISON Am J Physiol 100. 68, 1932
(133) COMROE, J H AND C F SCHMIDT Am J Physiol 138 536, 1943
(134) HARRISON, T R, W G HARRISON, J A CALHOUN AND J P MARSH Arch int Med 50 690, 1932
(135) HEYMANS, C AND J J BOUCKAERT J. Physiol 69 254, 1930
(136) GRAY, J S Fed Proc 5 35, 1946
(137) BARCROFT, J AND R MARGARIA J Physiol 72 175, 1931
(138) NIELSON, M Skand Arch Physiol 74 299, 1936
(139) GERARD, R W Physiol Rev 12 469, 1932
(140) GERARD, R W Ann Rev Physiol 4 329, 1942